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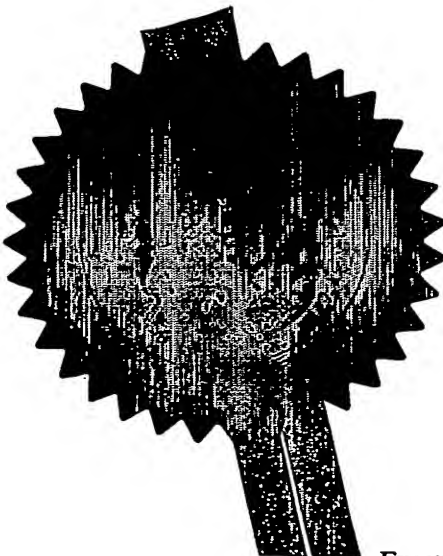
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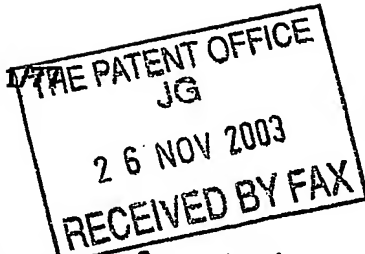
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Patents ADP number (if you know it)	8103517001		
If the applicant is a corporate body, give the country/state of its incorporation	United Kingdom		
4. Title of the invention	"Cancer Treatment"		
5. Name of your agent (if you have one)	Murgitroyd & Company		
"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)	Scotland House 165-169 Scotland Street Glasgow G5 8PL		
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Description

68

Claim(s)

6

Abstract

-

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18

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Cancer Treatment

Field of the Invention

The present invention relates to cancer treatment. In particular, it relates to assays and methods of determining susceptibility to resistance to anti-cancer drugs such as fluoropyrimidines, and methods and compositions for treatment of cancer.

Background to the Invention

5-FU⁴ is widely used in the treatment of a range of cancers including colorectal, breast and cancers of the aerodigestive tract. The mechanism of cytotoxicity of 5-FU has been ascribed to the misincorporation of fluoronucleotides into RNA and DNA and to the inhibition of the nucleotide synthetic enzyme thymidylate synthase (TS) (Longley et al., 2003). TS catalyses the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) with 5,10-methylene

1 tetrahydrofolate (CH₂THF) as the methyl donor. This
2 reaction provides the sole intracellular source of
3 thymidylate, which is essential for DNA synthesis
4 and repair. The 5-FU metabolite fluorodeoxyuridine
5 monophosphate (FdUMP) forms a stable complex with TS
6 and CH₂THF resulting in enzyme inhibition (Longley
7 et al., 2003). Recently, more specific folate-based
8 inhibitors of TS have been developed such as tomudex
9 (TDX) and Alimta (MTA), which form a stable complex
10 with TS and dUMP that inhibits binding of CH₂THF to
11 the enzyme (Hughes et al., 1999; Shih et al., 1997).
12 TS inhibition causes nucleotide pool imbalances that
13 result in S phase cell cycle arrest and apoptosis
14 (Aherne et al., 1996; Longley et al., 2002; Longley
15 et al., 2001). Oxaliplatin is a third generation
16 platinum-based DNA damaging agent that is used in
17 combination with 5-FU in the treatment of advanced
18 colorectal cancer (Giacchetti et al., 2000).

19
20 Drug resistance is a major factor limiting the
21 effectiveness of chemotherapies. Fas is a member of
22 the tumour necrosis factor (TNF) receptor family.
23 Binding of Fas Ligand (FasL) causes trimerization of
24 Fas and leads to recruitment of the adaptor protein
25 FADD (Fas-associated death domain), which in turn
26 recruits procaspase 8 zymogens to form the death-
27 inducing signalling complex (DISC) (Nagata, 1999).
28 Procaspase 8 molecules become activated at the DISC
29 and subsequently activate pro-apoptotic downstream
30 molecules such as caspase 3 and BID. FasL expression
31 is up-regulated in most colon tumours, and it has
32 been postulated that tumour FasL induces apoptosis

1 of Fas-sensitive immune effector cells (O'Connell et
2 al., 1999). This mechanism of immune escape requires
3 that tumour cells develop resistance to Fas-mediated
4 apoptosis to prevent autocrine and paracrine tumour
5 cell death.

6
7 A key inhibitor of Fas signaling is c-FLIP, which
8 inhibits procaspase 8 recruitment and processing at
9 the DISC (Krueger et al., 2001). Differential
10 splicing gives rise to long (c-FLIP_L) and short (c-
11 FLIP_S) forms of c-FLIP, both of which bind to FADD
12 within the DISC. c-FLIP_S directly inhibits caspase 8
13 activation at the DISC, whereas c-FLIP_L is first
14 cleaved to a p43 truncated form that inhibits
15 complete processing of procaspase 8 to its active
16 subunits. c-FLIP also inhibits procaspase 8
17 activation at DISCs formed by the TRAIL (TNF-related
18 apoptosis-inducing ligand) death receptors DR4
19 (TRAIL-R1) and DR5 (TRAIL-R2) (Krueger et al.,
20 2001). In addition to blocking caspase 8 activation,
21 DISC-bound c-FLIP has been reported to promote
22 activation of the ERK, PI3-kinase/Akt and NF-κB
23 signaling pathways (Krueger et al., 2001). Thus, c-
24 FLIP potentially converts death receptor signaling
25 from pro- to anti-apoptotic by activating intrinsic
26 survival pathways. Significantly, c-FLIP_L has been
27 found to be overexpressed in colonic adenocarcinomas
28 compared to matched normal tissue, suggesting that
29 c-FLIP may contribute to in vivo tumour
30 transformation (Ryu et al., 2001).

31
32

1 Summary of the Invention

2

3 As described herein and, as shown in our co-pending
4 GB patent application entitled "treatment
5 Medicament" and filed on the same day as the present
6 application, the present inventors have surprisingly
7 shown that by combining treatment using a death
8 receptor ligand, such as an anti FAS antibody, for
9 example, CH-11, with a chemotherapeutic agent such
10 as 5-FU or an antifolate drug, such as raltitrexed
11 (RTX) or pemetrexed (MTA, Alimta), a synergistic
12 effect is achieved in the killing of cancer cells.
13 However, the synergistic effect achieved was
14 abrogated in cancer cells which overexpress c-FLIP.

15

16 The demonstration that high levels of c-FLIP
17 expression in cancer cells inhibits drug induced
18 apoptosis of such cells enables the determination
19 prior to treatment of whether or not treatment with
20 a particular drug regime may be effective in a
21 particular patient. Thus, the present invention may
22 be used in assays to determine whether or not
23 treatment with a particular chemotherapeutic agent
24 may be effective in a particular patient.

25

26 Accordingly, in a first aspect of the present
27 invention, there is provided a method to predict
28 response of tumour cells to in vivo treatment with
29 a chemotherapeutic regime, said method comprising
30 the steps:

31 (a) providing an in vitro sample containing tumour
32 cells from a subject;

1 (b) determining the basal expression of one or more
2 of the genes encoding c-FLIP protein, wherein
3 enhanced expression of said gene correlates with
4 enhanced resistance to the chemotherapeutic regime.

5
6 Basal expression in the tumour cells may be compared
7 with basal expression in control samples. The
8 control samples may be 5-FU sensitive, oxaliplatin
9 sensitive and/or tomudex sensitive cancer cell-
10 lines. For example, the control sample may be the
11 H630 5-FU sensitive cancer cell line.

12
13 Alternatively, the control samples may be samples of
14 cells from non-cancerous tissues of human subjects,
15 preferably cancer-free human subjects. The basal
16 expression level of the gene(s) in the control
17 sample(s) may be determined in advance to provide
18 control basal expression level value(s) with which
19 to compare the expression level(s) of the in vitro
20 sample.

21
22 As well as showing that overexpression of basal c-
23 FLIP is associated with enhanced resistance to
24 chemotherapeutic regimes, for example, with enhanced
25 resistance to combined therapy comprising treatment
26 with anti-Fas ligand, for example, CH-11, combined
27 with a chemotherapeutic agent such as 5-FU or an
28 antifolate drug, the inventors have further shown
29 that basal expression of c-FLIP is enhanced in
30 certain tumour cells in response to treatment with a
31 chemotherapeutic regime.

32

1 Thus, in a second aspect of the present invention,
2 there is provided a method for evaluating in vitro
3 the response of tumour cells from a subject to the
4 presence of a chemotherapeutic regime to predict
5 response of the tumour cells in vivo to treatment
6 with the chemotherapeutic regime, which method
7 comprises:

- 8 (a) providing an in vitro sample containing tumour
9 cells from a subject;
10 (b) exposing a portion of said sample of tumour
11 cells to said chemotherapeutic regime;
12 (c) measuring expression of c-FLIP in said tumour
13 cells; wherein enhanced expression of c-FLIP in
14 response to said chemotherapeutic regime is
15 indicative of enhanced resistance to said
16 chemotherapeutic regime.

17
18 The presence of enhanced expression can be
19 determined, for example, with reference to
20 expression in a control portion of said sample which
21 has not been exposed to said chemotherapeutic regime
22 or to expression of said gene in the same sample
23 prior to application of the chemotherapeutic regime.
24

25 In preferred embodiments of the invention,
26 expression of c-FLIP in the sample exposed to said
27 chemotherapeutic agent is considered to be enhanced
28 if the expression is at least 2-fold, preferably at
29 least 3-fold, more preferably at least 4-fold, even
30 more preferably at least 5-fold, yet more preferably
31 at least 10-fold, most preferably at least 12-fold
32 that of c-FLIP in the control portion of said sample

1 which has not been exposed to said chemotherapeutic
2 regime.

3
4 The chemotherapeutic regime may be any
5 chemotherapeutic treatment suitable for treatment of
6 tumours. For example, the regime may include
7 treatment with one or more suitable chemotherapeutic
8 agents and/or one or more anti-tumour specific
9 binding members.

10
11 In one preferred embodiment, the chemotherapeutic
12 regime does not consist of treatment with 5-FU,
13 tomudex and/or oxaliplatin.

14
15 In particularly preferred embodiments of the
16 invention, the chemotherapeutic regime comprises
17 treatment using a death receptor ligand, such as an
18 anti FAS antibody, for example, CH-11, combined with
19 a chemotherapeutic agent such as 5-FU or an
20 antifolate drug, such as raltitrexed (RTX) or
21 pemetrexed (MTA, Alimta). As described herein, such
22 combinations are strongly synergistic.

23
24 Such a treatment regime forms an independent aspect
25 of the present invention.

26
27 As described in the Examples, in cell lines which
28 demonstrated overexpression of c-FLIP and associated
29 resistance to chemotherapy e.g 5-FU induced
30 apoptosis, inhibition of FLIP expression reversed
31 the resistance to chemotherapy -induced apoptosis.
32

1 Accordingly, in a third aspect, the invention
2 provides a method of sensitising cancer cells to
3 chemotherapy, said method comprising the step of
4 administration to said cells a c-FLIP inhibitor.

5

6 Any suitable c-FLIP inhibitor may be used in methods
7 of the invention. The inhibitor may be peptide or
8 non-peptide.

9

10 In one preferred embodiment, said c-FLIP inhibitor
11 is an antisense molecule which modulates the
12 expression of the gene encoding c-FLIP.

13

14 In a more preferred embodiment, said c-FLIP
15 inhibitor is an RNAi agent, which modulates
16 expression of the c-FLIP gene. The agent may be an
17 siRNA, an shRNA, a ddsRNAi construct or a
18 transcription template thereof, e.g., a DNA encoding
19 an shRNA. In preferred embodiments the RNAi agent
20 is an siRNA which is homologous to a part of the
21 mRNA sequence of the gene encoding c-FLIP.

22

23 Indeed such an RNAi agent represents a fourth
24 independent aspect of the present invention.

25

26 Preferred RNAi agents of and for use in the
27 invention are between 15 and 25 nucleotides in
28 length, preferably between 19 and 22 nucleotides,
29 most preferably 21 nucleotides in length. In
30 particularly preferred embodiments of the invention,
31 the RNAi agent has the nucleotide sequence shown as
32 SEQ ID NO: 1.

1

2 AAG CAG TCT GTT CAA GGA GCA (SEQ ID NO: 1)

3

4 According to a fifth aspect of the invention, there
5 is provided a vector comprising an RNAi agent of the
6 invention.

7

8 Furthermore, the invention may also be used to
9 identify novel c-FLIP inhibitors, which may be used
10 in the invention and which may be useful in
11 chemotherapeutic treatments and regimes. Such agents
12 may reduce or inhibit, either directly or
13 indirectly, the effects of c-FLIP.

14

15 Accordingly, in a sixth aspect of the invention,
16 there is provided an assay method for identifying a
17 chemotherapeutic agent for use in the treatment of
18 cancer, said method comprising the steps:
19 (a) providing a sample of tumour cells;
20 (b) exposing a portion of said sample to a candidate
21 chemotherapeutic agent;
22 (c) determining expression of c-FLIP in said sample
23 wherein a reduction in expression of c-FLIP compared
24 to expression in a control sample is indicative of
25 chemotherapeutic activity.

26

27 Expression in a control sample may be determined
28 with reference to a different sample of said tumour
29 cells which has not been exposed to said candidate
30 agent or with reference to expression in the same
31 sample prior to application of the candidate
32 chemotherapeutic agent.

10

1
2 C-FLIP inhibitors of and for use in the invention
3 may be used in *in vitro* and *in vivo* to kill cancer
4 cells.

5
6 Thus, in a seventh aspect, the present invention
7 provides a method of killing cancer cells comprising
8 administration of a therapeutically effective amount
9 of a c-FLIP inhibitor.

10
11 In an eighth aspect, the present invention provides
12 a method of treating cancer comprising
13 administration of a therapeutically effective amount
14 of a c-FLIP inhibitor.

15
16 As described above, a C-FLIP inhibitor may be used
17 to reverse or reduce resistance to chemotherapy-
18 induced apoptosis.

19
20 In a tenth aspect, there is provided the use of
21 a c-FLIP inhibitor in the preparation of a
22 medicament for treating cancer.

23
24 According to an eleventh aspect, there is provided a
25 pharmaceutical composition for the treatment of
26 cancer, wherein the composition comprises a c-FLIP
27 inhibitor and a pharmaceutically acceptable
28 excipient, diluent or carrier.

29
30
31 The c-FLIP inhibitor may be administered alone or in
32 combination with one or more further

1 chemotherapeutic substances. Such substances may be
2 chemotherapeutic agents as described above or may be
3 specific binding members with chemotherapeutic
4 activity.

5

6 In particularly preferred embodiments of the
7 invention, the c-FLIP inhibitor is administered as
8 part of a treatment regime comprising
9 (a) a c-FLIP inhibitor and
10 (b) (i) a specific binding member which binds to a
11 cell death receptor, or a nucleic acid encoding said
12 binding member; and
13 (ii) a chemotherapeutic agent.

14

15 Thus, in a preferred aspect of the tenth aspect of
16 the invention, there is provided the use of
17 (a) a c-FLIP inhibitor and
18 (b) (i) a specific binding member which binds to a
19 cell death receptor, or a nucleic acid encoding said
20 binding member; and/or
21 (ii) a chemotherapeutic agent in the preparation of
22 a medicament for treating cancer.

23

24 Further in a preferred aspect of the eleventh aspect
25 of the invention, there is provided a pharmaceutical
26 composition for the treatment of cancer, wherein the
27 composition comprises a) a c-FLIP inhibitor and
28 (b) (i) a specific binding member which binds to a
29 cell death receptor, or a nucleic acid encoding said
30 binding member; and/or
31 (ii) a chemotherapeutic agent and
32 (c) a pharmaceutically acceptable excipient, diluent

12

1 or carrier.

2

3 In an twelfth aspect, there is provided a product
4 comprising:

5 a) a c-FLIP inhibitor and

6 (b) (i) a specific binding member which binds to a
7 cell death receptor, or a nucleic acid encoding said
8 binding member; and/or

9 (ii) a chemotherapeutic agent

10 as a combined preparation for the simultaneous,
11 separate or sequential use in the treatment of
12 cancer.

13

14 In a thirteenth aspect, there is provided a kit for
15 the treatment of cancer, said kit comprising a) a c-
16 FLIP inhibitor and

17 (b) (i) a specific binding member which binds to a
18 cell death receptor, or a nucleic acid encoding said
19 binding member; and/or

20 (ii) a chemotherapeutic agent and

21 (c) instructions for the administration of (a) and

22 (b) separately, sequentially or simultaneously.

23

24 The c-FLIP inhibitor, the specific binding member
25 and/or the chemotherapeutic agent may be
26 administered simultaneously, sequentially or
27 simultaneously. In preferred embodiments of the
28 invention, the C-FLIP inhibitor is administered
29 prior to the specific binding member and the
30 chemotherapeutic agent.

31

1 A preferred binding member for use in the invention
2 is an antibody or a fragment thereof. In
3 particularly preferred embodiments, the binding
4 member is the FAS antibody CH11 (Yonehara, S.,
5 Ishii, A. and Yonehara, M. (1989) J. Exp. Med. 169,
6 1747-1756) (available commercially e.g. from Upstate
7 Biotechnology, Lake Placid, NY).

8
9 Any suitable chemotherapeutic agent may be used in
10 the present invention. In preferred embodiments, the
11 agent is doxorubicin, oxaliplatin, taxol, 5-
12 Fluorouracil (5-FU), Irinotecan (CPT11) or an
13 antifolate e.g. MTA or RTX. In one preferred
14 embodiment, the agent is, 5-Fluorouracil, an
15 antifolate (for example RTX or MTA), or a
16 combination thereof. In a particularly preferred
17 embodiment, the agent is 5-FU or an antifolate.
18 Preferably, the agent is an antifolate. In a
19 particularly preferred embodiment the agent is MTA.

20
21 In preferred embodiments of the invention, the c-
22 FLIP inhibitor is used in combination with a
23 specific binding member which binds to a cell death
24 receptor as described above, or a nucleic acid
25 encoding said binding member; and a chemotherapeutic
26 agent.

27
28 In those embodiments in which both are used, the
29 concentrations of binding members and
30 chemotherapeutic agents used are preferably
31 sufficient to provide a synergistic effect.

32

1 Synergism is preferably defined as an RI of greater
2 than unity using the method of Kern as modified by
3 Romaneli (13, 14). The RI may be calculated as the
4 ratio of expected cell survival (S_{exp} , defined as the
5 product of the survival observed with drug A alone
6 and the survival observed with drug B alone) to the
7 observed cell survival (S_{obs}) for the combination of
8 A and B ($RI = S_{exp}/S_{obs}$). Synergism may then be defined
9 as an RI of greater than unity.

10

11 In preferred embodiments of the invention, said
12 specific binding member and chemotherapeutic agent
13 are provided in concentrations sufficient to produce
14 an RI of greater than 1.5, more preferably greater
15 than 2.0, most preferably greater than 2.25.

16

17 The combined medicament thus preferably produces a
18 synergistic effect when used to treat tumour cells.

19

20 Preferred features of each aspect of the invention
21 are as for each of the other aspects mutatis
22 mutandis unless the context demands otherwise.

23

24 Detailed Description

25

26 As described above, the present invention relates to
27 methods of screening samples comprising tumour cells
28 for expression of particular genes in order to
29 determine suitability for treatment using
30 chemotherapeutic agents and methods of treatment of
31 cancer.

32

1 The methods of the invention may involve the
2 determination of expression of FLIP protein.

3
4 The expression of FLIP may be measured using any
5 technique known in the art. Either mRNA or protein
6 can be measured as a means of determining up-or down
7 regulation of expression of a gene. Quantitative
8 techniques are preferred. However semi-quantitative
9 or qualitative techniques can also be used. Suitable
10 techniques for measuring gene products include, but
11 are not limited to, SAGE analysis, DNA microarray
12 analysis, Northern blot,
13 Western blot, immunocytochemical analysis, and
14 ELISA.

15
16 In the methods of the invention, RNA can be detected
17 using any of the known techniques in the art.
18 Preferably an amplification step is used as the
19 amount of RNA from the sample may be very small.
20 Suitable techniques may include real-time RT-PCR,
21 hybridisation of copy mRNA (cRNA) to an array of
22 nucleic acid probes and Northern Blotting.

23
24 For example, when using mRNA detection, the method
25 may be carried out by converting the isolated mRNA
26 to cDNA according to standard methods; treating the
27 converted cDNA with amplification reaction reagents
28 (such as cDNA PCR reaction reagents) in a container
29 along with an appropriate mixture of nucleic acid
30 primers; reacting the contents of the container to
31 produce amplification products; and analyzing the
32 amplification products to detect the presence of

1 gene expression products of one or more of the genes
2 encoding FLIP protein. Analysis may be accomplished
3 using Southern Blot analysis to detect the presence
4 of the gene products in the amplification product.
5 Southern Blot analysis is known in the art. The
6 analysis step may be further accomplished by
7 quantitatively detecting the presence of such gene
8 products in the amplification products, and
9 comparing the quantity of product detected against a
10 panel of expected values for known presence or
11 absence in normal and malignant tissue derived using
12 similar primers.

13

14 In e.g. determining gene expression in carrying out
15 methods of the invention, conventional molecular
16 biological, microbiological and recombinant DNA
17 techniques techniques known in the art may be
18 employed. Details of such techniques are described
19 in, for example, Sambrook, Fritsch and Maniatis,
20 "Molecular Cloning, A Laboratory Manual, Cold
21 Spring Harbor Laboratory Press, 1989, and Ausubel et
22 al, Short Protocols in Molecular Biology, John Wiley
23 and Sons, 1992).

24

25 The methods of the invention may be used to
26 determine the suitability for treatment of any
27 suitable cancer with a chemotherapeutic regime. For
28 example the methods of the invention may be used to
29 determine the sensitivity or resistance to treatment
30 of cancers including, but not limited to,
31 gastrointestinal, breast, prostate, head and neck
32 cancers.

1
2 The nature of the tumour or cancer will determine
3 the nature of the sample which is to be used in the
4 methods of the invention. The sample may be, for
5 example, a sample from a tumour tissue biopsy, bone
6 marrow biopsy or circulating tumour cells in e.g.
7 blood. Alternatively, e.g. where the tumour is a
8 gastrointestinal tumour, tumour cells may be
9 isolated from faeces samples. Other sources of
10 tumour cells may include plasma, serum,
11 cerebrospinal fluid, urine, interstitial fluid,
12 ascites fluid etc.

13
14 For example, solid tumours may be collected in
15 complete tissue culture medium with antibiotics.
16 Cells may be manually teased from the tumour
17 specimen or, where necessary, are enzymatically
18 disaggregated by incubation with collagenase/DNAse
19 and suspended in appropriate media containing, for
20 example, human or animal sera.

21
22 In other embodiments, biopsy samples may be isolated
23 and frozen or fixed in fixatives such as formalin.
24 The samples may then be tested for expression levels
25 of genes at a later stage.

26 27 **Binding members**

28
29 In the context of the present invention, a "binding
30 member" is a molecule which has binding specificity
31 for another molecule, in particular a receptor,
32 preferably a death receptor. The binding member may

1 be a member of a pair of specific binding members.
2 The members of a binding pair may be naturally
3 derived or wholly or partially synthetically
4 produced. One member of the pair of molecules may
5 have an area on its surface, which may be a
6 protrusion or a cavity, which specifically binds to
7 and is therefore complementary to a particular
8 spatial and polar organisation of the other member
9 of the pair of molecules. Thus, the members of the
10 pair have the property of binding specifically to
11 each other. A binding member of the invention and
12 for use in the invention may be any moiety, for
13 example an antibody or ligand, which preferably can
14 bind to a death receptor.

15
16 The binding member may bind to any death receptor.
17 Death receptors include, Fas, TNFR, DR-3, DR-4 and
18 DR-5. In preferred embodiments of the invention, the
19 death receptor is FAS.

20
21 In preferred embodiments, the binding member
22 comprises at least one human constant region.

23 24 Antibodies

25
26 An "antibody" is an immunoglobulin, whether natural
27 or partly or wholly synthetically produced. The
28 term also covers any polypeptide, protein or peptide
29 having a binding domain which is, or is homologous
30 to, an antibody binding domain. These can be
31 derived from natural sources, or they may be partly
32 or wholly synthetically produced. Examples of

1 antibodies are the immunoglobulin isotypes and their
2 isotypic subclasses and fragments which comprise an
3 antigen binding domain such as Fab, scFv, Fv, dAb,
4 Fd; and diabodies.

5
6 A binding member for use in certain embodiments, the
7 invention may be an antibody such as a monoclonal or
8 polyclonal antibody, or a fragment thereof. The
9 constant region of the antibody may be of any class
10 including, but not limited to, human classes IgG,
11 IgA, IgM, IgD and IgE. The antibody may belong to
12 any sub class e.g. IgG1, IgG2, IgG3 and IgG4. IgG1
13 is preferred.

14
15 As antibodies can be modified in a number of ways,
16 the term "antibody" should be construed as covering
17 any binding member or substance having a binding
18 domain with the required specificity. Thus, this
19 term covers antibody fragments, derivatives,
20 functional equivalents and homologues of antibodies,
21 including any polypeptide comprising an
22 immunoglobulin binding domain, whether natural or
23 wholly or partially synthetic. Chimeric molecules
24 comprising an immunoglobulin binding domain, or
25 equivalent, fused to another polypeptide are
26 therefore included. Cloning and expression of
27 chimeric antibodies are described in EP-A-0120694
28 and EP-A-0125023.

29
30 Examples of such fragments which can be used in the
31 invention include the Fab fragment, the Fd fragment,
32 the Fv fragment, the dAb fragment (Ward, E.S. et

1 al., Nature 341:544-546 (1989)), F(ab')₂ fragments,
2 single chain Fv molecules (scFv), bispecific single
3 chain Fv dimers (PCT/US92/09965) and "diabodies",
4 multivalent or multispecific fragments constructed
5 by gene fusion (WO94/13804; P. Hollinger et al.,
6 Proc. Natl. Acad. Sci. USA 90:6444-6448 (1993)).

7
8 A fragment of an antibody or of a polypeptide for
9 use in the present invention generally means a
10 stretch of amino acid residues of at least 5 to 7
11 contiguous amino acids, often at least about 7 to 9
12 contiguous amino acids, typically at least about 9
13 to 13 contiguous amino acids, more preferably at
14 least about 20 to 30 or more contiguous amino acids
15 and most preferably at least about 30 to 40 or more
16 consecutive amino acids.

17
18 A "derivative" of such an antibody or polypeptide,
19 or of a fragment antibody means an antibody or
20 polypeptide modified by varying the amino acid
21 sequence of the protein, e.g. by manipulation of the
22 nucleic acid encoding the protein or by altering the
23 protein itself. Such derivatives of the natural
24 amino acid sequence may involve insertion, addition,
25 deletion and/or substitution of one or more amino
26 acids, preferably while providing a peptide having
27 death receptor, e.g. FAS neutralisation and/or
28 binding activity. Preferably such derivatives
29 involve the insertion, addition, deletion and/or
30 substitution of 25 or fewer amino acids, more
31 preferably of 15 or fewer, even more preferably of

1 10 or fewer, more preferably still of 4 or fewer and
2 most preferably of 1 or 2 amino acids only.

3
4 In preferred embodiments, the binding member is
5 humanised. Methods for making humanised antibodies
6 are known in the art e.g see U.S. Patent No.
7 5,225,539. A humanised antibody may be a modified
8 antibody having the hypervariable region of a
9 monoclonal antibody and the constant region of a
10 human antibody. Thus the binding member may
11 comprise a human constant region. The variable
12 region other than the hypervariable region may also
13 be derived from the variable region of a human
14 antibody and/or may also be derived from a
15 monoclonal antibody. In such case, the entire
16 variable region may be derived from murine
17 monoclonal antibody and the antibody is said to be
18 chimerised. Methods for making chimerised
19 antibodies are known in the art (e.g see U.S. Patent
20 Nos. 4,816,397 and 4,816,567).

21
22 It is possible to take monoclonal and other
23 antibodies and use techniques of recombinant DNA
24 technology to produce other antibodies or chimeric
25 molecules which retain the specificity of the
26 original antibody. Such techniques may involve
27 introducing DNA encoding the immunoglobulin variable
28 region, or the complementary determining regions
29 (CDRs), of an antibody to the constant regions, or
30 constant regions plus framework regions, of a
31 different immunoglobulin. See, for instance, EP-A-
32 184187, GB 2188638A or EP-A-239400. A hybridoma or

1 other cell producing an antibody may be subject to
2 genetic mutation or other changes, which may or may
3 not alter the binding specificity of antibodies
4 produced.

5
6 A typical antibody for use in the present invention
7 is a humanised equivalent of CH11 or any chimerised
8 equivalent of an antibody that can bind to the FAS
9 receptor and any alternative antibodies directed at
10 the FAS receptor that have been chimerised and can
11 be use in the treatment of humans. Furthermore, the
12 typical antibody is any antibody that can cross-
13 react with the extracellular portion of the FAS
14 receptor and either bind with high affinity to the
15 FAS receptor, be internalised with the FAS receptor
16 or trigger signalling through the FAS receptor.

17
18 **Production of Binding Members**

19
20 Binding members, which may be used in the present
21 invention may be generated wholly or partly by
22 chemical synthesis. The binding members can be
23 readily prepared according to well-established,
24 standard liquid or, preferably, solid-phase peptide
25 synthesis methods, general descriptions of which are
26 broadly available (see, for example, in J.M. Stewart
27 and J.D. Young, Solid Phase Peptide Synthesis, 2nd
28 edition, Pierce Chemical Company, Rockford, Illinois
29 (1984), in M. Bodanzsky and A. Bodanzsky, The
30 Practice of Peptide Synthesis, Springer Verlag, New
31 York (1984); and Applied Biosystems 430A Users
32 Manual, ABI Inc., Foster City, California), or they

1 may be prepared in solution, by the liquid phase
2 method or by any combination of solid-phase, liquid
3 phase and solution chemistry, e.g. by first
4 completing the respective peptide portion and then,
5 if desired and appropriate, after removal of any
6 protecting groups being present, by introduction of
7 the residue X by reaction of the respective carbonic
8 or sulfonic acid or a reactive derivative thereof.

9
10 Another convenient way of producing a binding member
11 suitable for use in the present invention is to
12 express nucleic acid encoding it, by use of nucleic
13 acid in an expression system. Thus the present
14 invention further provides the use of (a) nucleic
15 acid encoding a specific binding member which binds
16 to a cell death receptor and (b) a chemotherapeutic
17 agent in the preparation of a medicament for
18 treating cancer.

19
20 Nucleic acids of and/or for use in accordance with
21 the present invention may comprise DNA or RNA and
22 may be wholly or partially synthetic. In a preferred
23 aspect, nucleic acid for use in the invention codes
24 for a binding member of the invention as defined
25 above. The skilled person will be able to determine
26 substitutions, deletions and/or additions to such
27 nucleic acids which will still provide a binding
28 member suitable for use in the present invention.

29
30 Nucleic acid sequences encoding a binding member for
31 use with the present invention can be readily
32 prepared by the skilled person using the information

1 and references contained herein and techniques known
2 in the art (for example, see Sambrook, Fritsch and
3 Maniatis, "Molecular Cloning", A Laboratory Manual,
4 Cold Spring Harbor Laboratory Press, 1989, and
5 Ausubel et al, Short Protocols in Molecular Biology,
6 John Wiley and Sons, 1992), given the nucleic acid
7 sequences and clones available. These techniques
8 include (i) the use of the polymerase chain reaction
9 (PCR) to amplify samples of such nucleic acid, e.g.
10 from genomic sources, (ii) chemical synthesis, or
11 (iii) preparing cDNA sequences. DNA encoding
12 antibody fragments may be generated and used in any
13 suitable way known to those of skill in the art,
14 including by taking encoding DNA, identifying
15 suitable restriction enzyme recognition sites either
16 side of the portion to be expressed, and cutting out
17 said portion from the DNA. The portion may then be
18 operably linked to a suitable promoter in a standard
19 commercially available expression system. Another
20 recombinant approach is to amplify the relevant
21 portion of the DNA with suitable PCR primers.
22 Modifications to the sequences can be made, e.g.
23 using site directed mutagenesis, to lead to the
24 expression of modified peptide or to take account of
25 codon preferences in the host cells used to express
26 the nucleic acid.

27

28 The nucleic acid may be comprised as construct(s) in
29 the form of a plasmid, vector, transcription or
30 expression cassette which comprises at least one
31 nucleic acid as described above. The construct may
32 be comprised within a recombinant host cell which

1 comprises one or more constructs as above.
2 Expression may conveniently be achieved by culturing
3 under appropriate conditions recombinant host cells
4 containing the nucleic acid. Following production
5 by expression a specific binding member may be
6 isolated and/or purified using any suitable
7 technique, then used as appropriate.

8
9 Binding members-encoding nucleic acid molecules and
10 vectors for use in accordance with the present
11 invention may be provided isolated and/or purified,
12 e.g. from their natural environment, in
13 substantially pure or homogeneous form, or, in the
14 case of nucleic acid, free or substantially free of
15 nucleic acid or genes of origin other than the
16 sequence encoding a polypeptide with the required
17 function.

18
19 Systems for cloning and expression of a polypeptide
20 in a variety of different host cells are well known.
21 Suitable host cells include bacteria, mammalian
22 cells, yeast and baculovirus systems. Mammalian
23 cell lines available in the art for expression of a
24 heterologous polypeptide include Chinese hamster
25 ovary cells, HeLa cells, baby hamster kidney cells,
26 NSO mouse melanoma cells and many others. A common,
27 preferred bacterial host is E. coli.

28
29 The expression of antibodies and antibody fragments
30 in prokaryotic cells such as E. coli is well
31 established in the art. For a review, see for
32 example Plückthun, Bio/Technology 9:545-551 (1991).

1 Expression in eukaryotic cells in culture is also
2 available to those skilled in the art as an option
3 for production of a binding member, see for recent
4 review, for example Reff, Curr. Opinion Biotech.
5 4:573-576 (1993); Trill et al., Curr. Opinion
6 Biotech. 6:553-560 (1995).

7
8 Suitable vectors can be chosen or constructed,
9 containing appropriate regulatory sequences,
10 including promoter sequences, terminator sequences,
11 polyadenylation sequences, enhancer sequences,
12 marker genes and other sequences as appropriate.
13 Vectors may be plasmids, viral e.g. 'phage, or
14 phagemid, as appropriate. For further details see,
15 for example, Sambrook et al., Molecular Cloning: A
16 Laboratory Manual: 2nd Edition, Cold Spring Harbor
17 Laboratory Press (1989). Many known techniques and
18 protocols for manipulation of nucleic acid, for
19 example in preparation of nucleic acid constructs,
20 mutagenesis, sequencing, introduction of DNA into
21 cells and gene expression, and analysis of proteins,
22 are described in detail in Ausubel et al. eds.,
23 Short Protocols in Molecular Biology, 2nd Edition,
24 John Wiley & Sons (1992).

25
26 The nucleic acid may be introduced into a host cell
27 by any suitable means. The introduction may employ
28 any available technique. For eukaryotic cells,
29 suitable techniques may include calcium phosphate
30 transfection, DEAE-Dextran, electroporation,
31 liposome-mediated transfection and transduction
32 using retrovirus or other virus, e.g. vaccinia or,

1 for insect cells, baculovirus. For bacterial cells,
2 suitable techniques may include calcium chloride
3 transformation, electroporation and transfection
4 using bacteriophage.

5
6 Marker genes such as antibiotic resistance or
7 sensitivity genes may be used in identifying clones
8 containing nucleic acid of interest, as is well
9 known in the art.

10

11 The introduction may be followed by causing or
12 allowing expression from the nucleic acid, e.g. by
13 culturing host cells under conditions for expression
14 of the gene.

15

16 The nucleic acid may be integrated into the genome
17 (e.g. chromosome) of the host cell. Integration may
18 be promoted by inclusion of sequences which promote
19 recombination with the genome in accordance with
20 standard techniques. The nucleic acid may be on an
21 extra-chromosomal vector within the cell, or
22 otherwise identifiably heterologous or foreign to
23 the cell.

24

25 RNAi agents

26

27 As described herein, c-FLIP inhibitors for use in
28 the invention may be RNAi agents.

29

30 RNA interference (RNAi) or posttranscriptional gene
31 silencing (PTGS) is a process whereby double-
32 stranded RNA induces potent and specific gene

1 silencing. RNAi is mediated by RNA-induced silencing
2 complex (RISC), a sequence-specific, multicomponent
3 nuclease that destroys messenger RNAs homologous to
4 the silencing trigger. RISC is known to contain
5 short RNAs (approximately 22 nucleotides) derived
6 from the double-stranded RNA trigger.

7
8 In one aspect, the invention provides methods of
9 employing an RNAi agent to modulate expression,
10 preferably reducing expression of a target gene, c-
11 FLIP, in a mammalian, preferably human host. By
12 reducing expression is meant that the level of
13 expression of a target gene or coding sequence is
14 reduced or inhibited by at least about 2-fold,
15 usually by at least about 5-fold, e.g., 10-fold, 15-
16 fold, 20-fold, 50-fold, 100-fold or more, as
17 compared to a control. In certain embodiments, the
18 expression of the target gene is reduced to such an
19 extent that expression of the c-FLIP gene /coding
20 sequence is effectively inhibited. By modulating
21 expression of a target gene is meant altering, e.g.,
22 reducing, translation of a coding sequence, e.g.,
23 genomic DNA, mRNA etc., into a polypeptide, e.g.,
24 protein, product.

25
26 The RNAi agents that may be employed in preferred
27 embodiments of the invention are small ribonucleic
28 acid molecules (also referred to herein as
29 interfering ribonucleic acids), that are present in
30 duplex structures, e.g., two distinct
31 oligoribonucleotides hybridized to each other or a
32 single ribooligonucleotide that assumes a small

1 hairpin formation to produce a duplex structure.
2 Preferred oligoribonucleotides are ribonucleic
3 acids of not greater than 100 nt in length,
4 typically not greater than 75 nt in length. Where
5 the RNA agent is an siRNA, the length of the duplex
6 structure typically ranges from about 15 to 30 bp,
7 usually from about 20 and 29 bps, most preferably 21
8 bp. Where the RNA agent is a duplex structure of a
9 single ribonucleic acid that is present in a hairpin
10 formation, i.e., a shRNA, the length of the
11 hybridized portion of the hairpin is typically the
12 same as that provided above for the siRNA type of
13 agent or longer by 4-8 nucleotides.

14
15 In certain embodiments, instead of the RNAi agent
16 being an interfering ribonucleic acid, e.g., an
17 siRNA or shRNA as described above, the RNAi agent
18 may encode an interfering ribonucleic acid. In these
19 embodiments, the RNAi agent is typically a DNA that
20 encodes the interfering ribonucleic acid. The DNA
21 may be present in a vector.

22
23 The RNAi agent can be administered to the host using
24 any suitable protocol known in the art. For example,
25 the nucleic acids may be introduced into tissues or
26 host cells by viral infection, microinjection,
27 fusion of vesicles, particle bombardment, or
28 hydrodynamic nucleic acid administration.

29
30 DNA directed RNA interference (ddRNAi) is an RNAi
31 technique which may be used in the methods of the
32 invention. ddRNAi is described in U.S. 6,573,099 and

1 GB 2353282. ddRNAi is a method to trigger RNAi
2 which involves the introduction of a DNA construct
3 into a cell to trigger the production of double
4 stranded (dsRNA), which is then cleaved into small
5 interfering RNA (siRNA) as part of the RNAi process.
6 ddRNAi expression vectors generally employ RNA
7 polymerase III promoters (e.g. U6 or H1) for the
8 expression of siRNA target sequences transfected in
9 mammalian cells. siRNA target sequences generated
10 from a ddRNAi expression cassette system can be
11 directly cloned into a vector that does not contain
12 a U6 promoter. Alternatively short single stranded
13 DNA oligos containing the hairpin siRNA target
14 sequence can be annealed and cloned into a vector
15 downstream of the pol-III promoter. The primary
16 advantages of ddRNAi expression vectors is that they
17 allow for long term interference effects and
18 minimise the natural interferon response in cells..
19

20 Antisense nucleic acids

21

22 As described herein, c-FLIP inhibitors for use in
23 the invention may be anti-sense molecules or nucleic
24 acid constructs that express such anti-sense
25 molecules as RNA. The antisense molecules may be
26 natural or synthetic. Synthetic antisense molecules
27 may have chemical modifications from native nucleic
28 acids. The antisense sequence is complementary to
29 the mRNA of the targeted c-FLIP gene, and inhibits
30 expression of the targeted gene products. Antisense
31 molecules inhibit gene expression through various
32 mechanisms, e.g. by reducing the amount of mRNA

1 available for translation, through activation of
2 RNase H, or steric hindrance. One or a combination
3 of antisense molecules may be administered, where a
4 combination may comprise multiple different
5 sequences.

6
7 Antisense molecules may be produced by expression of
8 all or a part of the c-FLIP sequence in an
9 appropriate vector, where the transcriptional
10 initiation is oriented such that an antisense strand
11 is produced as an RNA molecule. Alternatively, the
12 antisense molecule may be a synthetic
13 oligonucleotide. Antisense oligonucleotides will
14 generally be at least about 7, usually at least
15 about 12, more usually at least about 16 nucleotides
16 in length, and usually not more than about 50,
17 preferably not more than about 35 nucleotides in
18 length.

19
20 A specific region or regions of the endogenous c-
21 FLIP sense strand mRNA sequence is chosen to be
22 complemented by the antisense sequence. Selection of
23 a specific sequence for the oligonucleotide may use
24 an empirical method, where several candidate
25 sequences are assayed for inhibition of expression
26 of the target gene in an in vitro or animal model. A
27 combination of sequences may also be used, where
28 several regions of the mRNA sequence are selected
29 for antisense complementation.

30
31 Antisense oligonucleotides may be chemically
32 synthesized by methods known in the art (see Wagner

1 et al. (1993), supra, and Milligan et al., supra.)
2 Preferred oligonucleotides are chemically modified
3 from the native phosphodiester structure, in order
4 to increase their intracellular stability and
5 binding affinity. A number of such modifications
6 have been described in the literature, which alter
7 the chemistry of the backbone, sugars or
8 heterocyclic bases. Among useful changes in the
9 backbone chemistry are phosphorodiamidate linkages,
10 methylphosphonates phosphorothioates;
11 phosphorodithioates, where both of the non-bridging
12 oxygens are substituted with sulfur;
13 phosphoroamidites; alkyl phosphotriesters and
14 boranophosphates. Achiral phosphate derivatives
15 include 3'-O-5'-S-phosphorothioate, 3'-S-5'-O-
16 phosphorothioate, 3'-CH₂-5'-O-phosphonate and 3'-NH-
17 5'-O-phosphoroamidate. Peptide nucleic acids may
18 replace the entire ribose phosphodiester backbone
19 with a peptide linkage. Sugar modifications may also
20 be used to enhance stability and affinity.

21

22 Chemotherapeutic Agents

23

24 Any suitable chemotherapeutic agent or agents may be
25 used in the present invention. For example, an agent
26 for use in the invention may include but is not
27 limited to: 5-Fluorouracil (5 FU), antifolates, for
28 example RTX or MTA, Doxorubicin, taxol, Leucovorin,
29 Irinotecan, Mitomycin C, Oxaliplatin, Raltitrexed,
30 Tamoxifen or Cisplatin.

31

1 In particularly preferred embodiments, the agent is
2 5-FU or an antifolate. More preferably, the agent
3 is an antifolate. In one preferred embodiment, the
4 agent is MTA.

5

6 Treatment

7

8 "Treatment" includes any regime that can benefit a
9 human or non-human animal. The treatment may be in
10 respect of an existing condition or may be
11 prophylactic (preventative treatment). Treatment may
12 include curative, alleviation or prophylactic
13 effects.

14

15 "Treatment of cancer" includes treatment of
16 conditions caused by cancerous growth and includes
17 the treatment of neoplastic growths or tumours.
18 Examples of tumours that can be treated using the
19 invention are, for instance, sarcomas, including
20 osteogenic and soft tissue sarcomas, carcinomas,
21 e.g., breast-, lung-, bladder-, thyroid-, prostate-,
22 colon-, rectum-, pancreas-, stomach-, liver-,
23 uterine-, cervical and ovarian carcinoma, lymphomas,
24 including Hodgkin and non-Hodgkin lymphomas,
25 neuroblastoma, melanoma, myeloma, Wilms tumor, and
26 leukemias, including acute lymphoblastic leukaemia
27 and acute myeloblastic leukaemia, gliomas and
28 retinoblastomas.

29

30 In preferred embodiments of the invention, the
31 cancer is one or more of colorectal, breast ,

1 ovarian, cervical, gastric, lung, liver, skin and
2 myeloid (e.g. bone marrow) cancer.

3

4 Administration

5

6 As described above, c-FLIP inhibitors of and for use
7 in the present invention may be administered in any
8 suitable way. Moreover they may be used in
9 combination therapy with other treatments, for
10 example, other chemotherapeutic agents or binding
11 members. In such embodiments, the c-FLIP inhibitors
12 or compositions of the invention may be administered
13 simultaneously, separately or sequentially with
14 another chemotherapeutic agent.

15

16 Where administered separately or sequentially, they
17 may be administered within any suitable time period
18 e.g. within 1, 2, 3, 6, 12, 24, 48 or 72 hours of
19 each other. In preferred embodiments, they are
20 administered within 6, preferably within 2, more
21 preferably within 1, most preferably within 20
22 minutes of each other.

23

24 In a preferred embodiment, the c-FLIP inhibitors
25 and/or compositions of the invention are
26 administered as a pharmaceutical composition, which
27 will generally comprise a suitable pharmaceutical
28 excipient, diluent or carrier selected dependent on
29 the intended route of administration.

30

1 The c-FLIP inhibitors and/or compositions of the
2 invention may be administered to a patient in need
3 of treatment via any suitable route.

4
5 Some suitable routes of administration include (but
6 are not limited to) oral, rectal, nasal, topical
7 (including buccal and sublingual), vaginal or
8 parenteral (including subcutaneous, intramuscular,
9 intravenous, intradermal, intrathecal and epidural)
10 administration. Intravenous administration is
11 preferred.

12
13 The C-FLIP inhibitor, product or composition may be
14 administered in a localised manner to a tumour site
15 or other desired site or may be delivered in a
16 manner in which it targets tumour or other cells.
17 Targeting therapies may be used to deliver the
18 active agents more specifically to certain types of
19 cell, by the use of targeting systems such as
20 antibody or cell specific ligands. Targeting may be
21 desirable for a variety of reasons, for example if
22 the agent is unacceptably toxic, or if it would
23 otherwise require too high a dosage, or if it would
24 not otherwise be able to enter the target cells.

25
26 For intravenous, injection, or injection at the site
27 of affliction, the active ingredient will be in the
28 form of a parenterally acceptable aqueous solution
29 which is pyrogen-free and has suitable pH,
30 isotonicity and stability. Those of relevant skill
31 in the art are well able to prepare suitable
32 solutions using, for example, isotonic vehicles such

1 as Sodium Chloride Injection, Ringer's Injection,
2 Lactated Ringer's Injection. Preservatives,
3 stabilisers, buffers, antioxidants and/or other
4 additives may be included, as required.
5
6 Pharmaceutical compositions for oral administration
7 may be in tablet, capsule, powder or liquid form. A
8 tablet may comprise a solid carrier such as gelatin
9 or an adjuvant. Liquid pharmaceutical compositions
10 generally comprise a liquid carrier such as water,
11 petroleum, animal or vegetable oils, mineral oil or
12 synthetic oil. Physiological saline solution,
13 dextrose or other saccharide solution or glycols
14 such as ethylene glycol, propylene glycol or
15 polyethylene glycol may be included.
16
17 The c-FLIP inhibitors and/or compositions of the
18 invention may also be administered via microspheres,
19 liposomes, other microparticulate delivery systems
20 or sustained release formulations placed in certain
21 tissues including blood. Suitable examples of
22 sustained release carriers include semipermeable
23 polymer matrices in the form of shared articles,
24 e.g. suppositories or microcapsules. Implantable or
25 microcapsular sustained release matrices include
26 polylactides (US Patent No. 3, 773, 919; EP-A-
27 0058481) copolymers of L-glutamic acid and gamma
28 ethyl-L-glutamate (Sidman et al, Biopolymers 22(1):
29 547-556, 1985), poly (2-hydroxyethyl-methacrylate)
30 or ethylene vinyl acetate (Langer et al, J. Biomed.
31 Mater. Res. 15: 167-277, 1981, and Langer, Chem.
32 Tech. 12:98-105, 1982). Liposomes containing the

1 polypeptides are prepared by well-known methods: DE
2 3,218, 121A; Epstein et al, PNAS USA, 82: 3688-3692,
3 1985; Hwang et al, PNAS USA, 77: 4030-4034, 1980;
4 EP-A-0052522; E-A-0036676; EP-A-0088046; EP-A-
5 0143949; EP-A-0142541; JP-A-83-11808; US Patent Nos
6 4,485,045 and 4,544,545. Ordinarily, the liposomes
7 are of the small (about 200-800 Angstroms)
8 unilamellar type in which the lipid content is
9 greater than about 30 mol. % cholesterol, the
10 selected proportion being adjusted for the optimal
11 rate of the polypeptide leakage.

12
13 Examples of the techniques and protocols mentioned
14 above and other techniques and protocols which may
15 be used in accordance with the invention can be
16 found in Remington's Pharmaceutical Sciences, 16th
17 edition, Oslo, A. (ed), 1980.

18
19

20 **Pharmaceutical Compositions**

21

22 As described above, the present invention extends to
23 a pharmaceutical composition for the treatment of
24 cancer, the composition comprising a) a c-FLIP
25 inhibitor b) a pharmaceutically acceptable
26 excipient, diluent or carrier.

27

28 Pharmaceutical compositions according to the present
29 invention, and for use in accordance with the
30 present invention may comprise, in addition to
31 active ingredients, a pharmaceutically acceptable
32 excipient, carrier, buffer stabiliser or other

1 materials well known to those skilled in the art.
2 Such materials should be non-toxic and should not
3 interfere with the efficacy of the active
4 ingredient. The precise nature of the carrier or
5 other material will depend on the route of
6 administration, which may be oral, or by injection,
7 e.g. intravenous.

8
9 The formulation may be a liquid, for example, a
10 physiologic salt solution containing non-phosphate
11 buffer at pH 6.8-7.6, or a lyophilised powder.

12
13 **Dose**

14
15 The c-FLIP inhibitors or compositions of the
16 invention are preferably administered to an
17 individual in a "therapeutically effective amount",
18 this being sufficient to show benefit to the
19 individual. The actual amount administered, and
20 rate and time-course of administration, will depend
21 on the nature and severity of what is being treated.
22 Prescription of treatment, e.g. decisions on dosage
23 etc, is ultimately within the responsibility and at
24 the discretion of general practitioners and other
25 medical doctors, and typically takes account of the
26 disorder to be treated, the condition of the
27 individual patient, the site of delivery, the method
28 of administration and other factors known to
29 practitioners.

30

1 The invention will now be described further in the
2 following non-limiting examples. Reference is made
3 to the accompanying drawings in which:

4
5 Figure 1A illustrates Western blot analysis of Fas,
6 FasL, procaspase 8, FADD, BID, Bcl-2, c-FLIP_L, c-
7 FLIP_S, DcR3 and β -tubulin in MCF-7 cells 72 hours
8 after treatment with 5 μ M 5-FU and 50nM TDX.

9
10 Figure 1B illustrates analysis of the interaction
11 between Fas and FasL following treatment with 5 μ M 5-
12 FU and 50nM TDX for 48 hours. Lysates were
13 immunoprecipitated using a FasL polyclonal antibody
14 and analysed by Western blot using a Fas monoclonal
15 antibody.

16
17 Figure 1C illustrates analysis of the interaction
18 between Fas and p43- c-FLIP_L following treatment
19 with 5 μ M 5-FU and 50nM TDX for 48 hours. Lysates
20 were immunoprecipitated using the anti-Fas CH-11
21 monoclonal antibody and analysed by Western blot
22 using a c-FLIP monoclonal antibody.

23
24 Figure 2A illustrates flow cytometry of MCF-7 cells
25 treated with no drug (control), CH-11 alone
26 (250ng/ml), 5-FU alone (5 μ M) for 96 hours, or co-
27 treated with 5-FU for 72 hours followed by CH-11 for
28 a further 24 hours.

29
30 Figure 2B illustrates flow cytometry of MCF-7 cells
31 treated with no drug (control), CH-11 alone
32 (250ng/ml), TDX alone (50nM) for 96 hours, or co-

1 treated with TDX for 72 hours followed by CH-11 for
2 a further 24 hours.

3

4 Figure 2C illustrates Western blot analysis of Fas
5 expression in MCF-7 cells treated with 5 μ M 5-FU for
6 48 hours. β -tubulin was assessed as a loading
7 control.

8

9 Figure 2D illustrates flow cytometry of MCF-7 cells
10 treated with no drug (control), CH-11 alone
11 (250ng/ml), OXA alone (5 μ M) for 96 hours, or co-
12 treated with OXA for 72 hours followed by CH-11 for
13 a further 24 hours.

14

15 Figure 2E illustrates Western blot analysis of Fas,
16 procaspase 8 and PARP expression in MCF-7 cells
17 treated with 5 μ M 5-FU alone for 96 hours, or co-
18 treated with 5-FU for 72 hours followed by CH-11 for
19 a further 24 hours.

20

21 Figure 2F illustrates Western blot analysis
22 examining the kinetics of caspase 8 activation and
23 c-FLIP_L processing in MCF-7 cells treated for 72
24 hours with 5 μ M 5-FU followed by 250ng/ml CH-11 for
25 the indicated times.

26

27 Figure 3A illustrates Western blot analysis of Fas
28 expression in HCT116 cells treated with 5-FU, TDX or
29 OXA for 48 hours. Equal loading was assessed using a
30 β -tubulin antibody.

31

1 Figure 3B illustrates Western blot analysis of
2 procaspase 8 and PARP expression in HCT116 cells
3 treated no drug (Con), 5 μ M 5-FU, 100nM TDX or 2 μ M
4 OXA in the presence or absence of co-treatment with
5 200ng/ml CH-11. For each combined treatment the
6 cells were pre-treated with chemotherapeutic drug
7 for 24 hours followed by CH-11 for a further 24
8 hours.

9
10 Figure 4A illustrates Western blot of c-FLIP_L
11 expression in MCF-7 cells stably transfected with a
12 FLIPL (FL) construct or empty vector (EV).

13
14 Figure 4B illustrates MTT cell viability assays in
15 EV68, FL44 and FL64 cells treated with 5 μ M 5-FU in
16 combination with 250ng/ml CH-11. The combined
17 treatment resulted in a synergistic decrease in cell
18 viability in EV68 cells (RI=2.06), but not FL44
19 (RI=1.14) or FL64 (1.01) cells.

20
21 Figure 4C illustrates Western blot analysis of c-
22 FLIP_L, procaspase 8 and PARP expression in EV68 and
23 FL64 cells treated with no drug (Con) or 5 μ M 5-FU in
24 the presence (+) or absence (-) of co-treatment with
25 250ng/ml CH-11. For each combined treatment, the
26 cells were pre-treated with 5-FU for 72 hours
27 followed by CH-11 for a further 24 hours.

28
29 Figure 5A illustrates MTT cell viability assays in
30 EV68, FL44 and FL64 cells treated with 50nM TDX or
31 500nM MTA in the presence and absence of 250ng/ml
32 CH-11. Combined TDX/CH-11 treatment resulted in a

1 synergistic decrease in cell viability in EV68 cells
2 (RI=1.75), that was significantly reduced in FL44
3 (RI=1.22) or FL64 (RI=1.19) cells. Combined MTA/CH-
4 11 treatment resulted in a synergistic decrease in
5 cell viability in EV68 cells (RI=1.86), that was
6 significantly reduced in FL44 (RI=1.29) and FL64
7 (RI=1.06) cells.

8
9 Figure 5B illustrates MTT cell viability assays in
10 EV68, FL44 and FL64 cells treated with 2.5 μ M OXA in
11 the presence and absence of 250ng/ml CH-11. Combined
12 OXA/CH-11 treatment resulted in a synergistic
13 decrease in cell viability in EV68 cells (RI=2.13),
14 that was significantly reduced in FL64 (RI=1.22) or
15 FL44 (1.19) cells.

16
17 Figure 5C Western blot analysis of procaspase 8 and
18 PARP expression in EV68 and FL64 cells treated with
19 50nM TDX or 500nM MTA in the presence (+) or absence
20 (-) of co-treatment with 250ng/ml CH-11.

21
22 Figure 5D illustrates Western blot analysis of
23 procaspase 8 and PARP expression in EV68 and FL64
24 cells treated with 2.5 μ M OXA in the presence (+) or
25 absence (-) of co-treatment with 250ng/ml CH-11. For
26 each combined treatment, the cells were pre-treated
27 with 5-FU for 72 hours followed by CH-11 for a
28 further 24 hours.

29
30 Figure 6A illustrates c-FLIP_L and c-FLIP_S expression
31 in HCT116 cells transfected with 0, 1 and 10nM FLIP-

1 targeted siRNA for 48 hours. Equal loading was
2 assessed using a β -tubulin antibody.

3
4 Figure 6B illustrates MTT cell viability assays of
5 HCT116 cells transfected with 5nM FLIP-targeted (FT)
6 or scrambled control (SC) siRNA in the presence and
7 absence of co-treatment with 5 μ M 5-FU. Combined
8 treatment with 5-FU and FT siRNA resulted in a
9 synergistic decrease in cell viability (RI=1.92,
10 $p<0.0005$). No synergistic decrease in viability was
11 observed in cells co-treated with 5-FU and SC siRNA
12 (RI=0.98).

13
14 Figure 6C illustrates Western blot analysis of
15 caspase 8 activation and PARP cleavage in HCT116
16 cells 48 hours after treatment with no drug, 5 μ M 5-
17 FU or 100nM TDX in mock transfected cells (M), cells
18 transfected with 1nM scrambled control (SC) and
19 cells transfected with 1nM FLIP-targeted (FT) siRNA.

20
21 Figure 7A illustrates c-FLIP_L and c-FLIP_S expression
22 in MCF-7 cells transfected with 10nM FLIP-targeted
23 (FT) or scrambled control (SC) siRNA for 48 hours.
24 Equal loading was assessed using a β -tubulin
25 antibody.

26
27 Figure 7B illustrates MTT cell viability assays of
28 MCF-7 cells transfected with 2.5nM FT siRNA in the
29 presence and absence of co-treatment with 5 μ M 5-FU.
30 The combined treatment resulted in a synergistic
31 decrease in cell viability (RI=1.56, $p<0.005$).

1 Figure 7C Western blot analysis of PARP cleavage in
2 MCF-7 cells 96 hours after treatment with 5-FU in
3 the presence (+) and absence (-) of 10nM FLIP-
4 targeted siRNA:
5

6 Figure 8 illustrates MTT cell viability assays of
7 HCT116 cells transfected with 0.5nM FT or SC siRNA
8 in the presence and absence of co-treatment with:
9 Fig 8A 5 μ M 5-FU; Fig 8B 100nM TDX and Fig 8C 1 μ M
10 OXA. Cells were assayed after 72 hours. Combined
11 treatment with FT siRNA (but not SC siRNA) and each
12 cytotoxic drug resulted in synergistic decreases in
13 cell viability as indicated by the RI values
14 ($p < 0.0005$ for each combination).
15

16 Examples

17

18 MATERIALS AND METHODS

19 Cell Culture. All cells were maintained in 5% CO₂ at
20 37°C. MCF-7 cells were maintained in DMEM with 10%
21 dialyzed bovine calf serum supplemented with 1mM
22 sodium pyruvate, 2mM L-glutamine and 50 μ g/ml
23 penicillin/streptomycin (from Life Technologies
24 Inc., Paisley, Scotland). HCT116 cells were grown in
25 McCoy's 5A medium (GIBCO) supplemented with 10%
26 dialysed foetal calf serum, 50mg/ml penicillin-
27 streptomycin, 2mM L-glutamine and 1mM sodium
28 pyruvate. Stably transfected MCF-7 and HCT116 cell
29 lines and 'mixed populations' of transfected cells
30 were maintained in medium supplemented with 100 μ g/ml
31 (MCF-7) or 1.5mg/ml (HCT116) G418 (from Life
32 Technologies Inc).

1 Western Blotting. Western blots were performed as
2 previously described (Longley et al., 2002). The
3 Fas/CD95, Bcl-2 and BID (Santa Cruz Biotechnology,
4 Santa Cruz, CA), caspase 8 (Oncogene Research
5 Products, Darmstadt, Germany), PARP (PharMingen, BD
6 Biosciences, Oxford, England), c-FLIP (NF-6, Alexis,
7 Bingham UK) DoR3 (Imgenex, San Diego, CA) mouse
8 monoclonal antibodies were used in conjunction with
9 a horseradish peroxidase (HRP)-conjugated sheep
10 anti-mouse secondary antibody (Amersham, Little
11 Chalfont, Buckinghamshire, England). FasL rabbit
12 polyclonal antibody (Santa Cruz Biotechnology) was
13 used in conjunction with an HRP-conjugated donkey
14 anti-rabbit secondary antibody (Amersham). Equal
15 loading was assessed using a β -tubulin mouse
16 monoclonal primary antibody (Sigma).

17

18 Co-immunoprecipitation reactions. 250 μ l of Protein A
19 (IgG) or Protein L (IgM) Sepharose beads (Sigma) and
20 1 μ g of the appropriate antibody were mixed at 4°C
21 for 1 hour. Antibody-associated beads were washed
22 three times with ELB buffer (250mM NaCl, 0.1%
23 IPEGAL, 5mM EDTA, 0.5mM DTT, 50mM HEPES). Protein
24 lysate (200-400 μ g) was then added, and the mixture
25 rotated at 4°C for 1 hour. The beads were then
26 washed in ELB buffer five times and resuspended in
27 100 μ l of Western sample buffer (250mM TRIS pH 6.8,
28 4% SDS, 2% glycerol, 0.02% bromophenol blue)
29 containing 10% β -mercaptoethanol. The samples were
30 then heated at 95°C for 5 minutes and centrifuged
31 (5mins/4,000rpm/4°C). The supernatant was collected
32 and analysed by Western blotting.

1
2 Cell Viability Assays. Cell viability was assessed
3 by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-
4 diphenyltetrazolium bromide, Sigma) assay (Mosmann,
5 1983). To investigate drug-induced Fas-mediated
6 apoptosis, cells were seeded at 2,000-5,000 cells
7 per well on 96-well plates. After 24 hours, the
8 cells were treated with a range of concentrations of
9 5-FU, TDX, MTA or OXA for 24-72 hours followed by
10 the agonistic Fas monoclonal antibody, CH-11 (MBL,
11 Watertown, MA) for a further 24-48 hours. To assess
12 chemotherapy/siRNA interactions, 20,000-50,000 cells
13 were seeded per well on 24-well plates. Twenty-four
14 hours later, the cells were transfected with FLIP-
15 targeted (FT) or scrambled siRNA (SC). Four hours
16 after transfection, the cells were treated with a
17 range of concentrations of each drug for a further
18 72-96 hours. MTT (0.5mg/ml) was added to each well
19 and the cells were incubated at 37°C for a further 2
20 hours. The culture medium was removed and formazan
21 crystals reabsorbed in 200µl (96-well) or 1ml (24-
22 well) DMSO. Cell viability was determined by reading
23 the absorbance of each well at 570nm using a
24 microplate reader (Molecular Devices, Wokingham,
25 England).
26
27 Flow Cytometric Analysis. Cells were seeded at 1×10^5
28 per well of a 6-well tissue culture plate. After 24
29 hours, 5-FU, TDX or OXA was added to the medium and
30 the cells cultured for a further 72 hours, after
31 which time 250ng/ml CH-11 was added for 24 hours.
32 DNA content of harvested cells was evaluated after

1 propidium iodide staining of cells using the EPICS
2 XL Flow Cytometer (Coulter, Miami, FL).

3
4 siRNA transfections. FLIP-targeted siRNA was
5 designed using the Ambion siRNA target finder and
6 design tool
7 (www.ambion.com/techlib/misc/siRNA_finder.html) to
8 inhibit both splice variants of c-FLIP. Both c-FLIP-
9 targeted (FT) and scrambled control (SC) siRNA were
10 obtained from Xeragon (Germantown, MD). The FT siRNA
11 sequence used was: AAG CAG TCT GTT CAA GGA GCA. The
12 SC siRNA sequence used was: AAT TCT CCG AAC GTG TCA
13 CGT. siRNA transfections were performed on sub-
14 confluent cells incubated in Optimem medium using
15 the oligofectamine reagent (both from Life
16 Technologies Inc) according to the manufacturer's
17 instructions.

18
19 Statistical Analyses. The nature of the interaction
20 between the chemotherapeutic drugs and CH-11 was
21 determined by calculating the R index (RI), which
22 was initially described by Kern and later modified
23 by Romanelli (Kern et al., 1988; Romanelli et al.,
24 1998). The RI is calculated as the ratio of expected
25 cell survival (Sexp, defined as the product of the
26 survival observed with drug A alone and the survival
27 observed with drug B alone) to the observed cell
28 survival (Sobs) for the combination of A and B
29 ($RI = S_{exp} / S_{obs}$). Synergism is then defined as an RI
30 of greater than unity. Romanelli et al suggest
31 that a synergistic interaction may be of
32 pharmacological interest when RI values are around

1 2.0 (Romanelli et al., 1998). To further assess the
2 statistical significance of the interactions, we
3 designed a univariate ANOVA analysis using the SPSS
4 software package. This was an additive model based
5 on the null hypothesis that there was no interaction
6 between the drugs.

7

8 RESULTS

9

10 c-FLIP_L is up-regulated, processed and bound to Fas
11 in response to 5-FU and TDX. Analysis of Fas
12 expression in MCF-7 cells revealed that it was up-
13 regulated by ~12-fold 72 hours after treatment with
14 an IC60 dose 5-FU and was also highly up-regulated
15 (by ~7-fold) in response to treatment with an IC60
16 dose (25nM) of TDX (Fig. 1A). FasL expression was
17 unaffected by each drug treatment, but appeared to
18 be highly expressed in these cells. Expression of
19 FADD was also unaffected by drug treatment. Somewhat
20 surprisingly, neither caspase 8, nor its substrate
21 BID were activated in 5-FU- or TDX-treated cells as
22 indicated by a lack of down-regulation of the levels
23 of procaspase 8 or full-length BID (Fig. 1A). Bcl-2
24 was highly down-regulated in response to each agent.
25 Interestingly, c-FLIP_L but not c-FLIP_S was up-
26 regulated by drug treatment. Furthermore, c-FLIP_L
27 was processed to its p43-form indicative of its
28 recruitment and processing at the DISC (Fig. 1A).
29 Expression of the Fas decoy receptor Dcr3 was
30 unaltered by drug treatment in these cells.

31

1 To further investigate the apparent inhibition of
2 caspase 8 activation in 5-FU- and TDX-treated cells,
3 we analysed the interaction between Fas and FasL
4 following drug treatment. Co-immunoprecipitation
5 reactions demonstrated that there was increased Fas-
6 FasL binding following drug treatment (Fig. 1B),
7 suggesting that the inhibition of caspase 8
8 activation was occurring downstream of receptor
9 ligation. In support of this, we found that drug
10 treatment increased the interaction between Fas and
11 p43- c-FLIP_L (Fig. 1C). These results suggested the
12 involvement of c-FLIP_L in inhibiting drug-induced
13 activation of Fas-mediated apoptosis in MCF-7 cells.

14
15 Activation of drug-induced apoptosis by the Fas-
16 targeted antibody CH-11 coincides with processing of
17 c-FLIP_L. Expression of FasL by activated T cells and
18 NK cells induces apoptosis of Fas expressing target
19 cells in vivo. To mimic the effects of these immune
20 effector cells in vitro, the agonistic Fas
21 monoclonal antibody CH-11 was used. Cells were
22 treated with either 5-FU or TDX for 72 hours
23 followed by 250ng/ml CH-11 treatment for 24 hours.
24 We found that CH-11 alone had little effect on
25 apoptosis (Figs. 2A and B). Treatment with 5-FU
26 alone for 96 hours resulted in a modest ~2-fold
27 induction of apoptosis in response to 5µM 5-FU (Fig.
28 2A). However, addition of CH-11 to 5-FU-treated
29 cells resulted in a dramatic increase in apoptosis,
30 with a ~55% of cells in the sub-G1/G0 apoptotic
31 phase following co-treatment with 5µM 5-FU and CH-
32 11. Similarly, the combination of TDX with CH-11

1 resulted in dramatic activation of apoptosis, with
2 ~60% of cells in the sub-G1/G0 apoptotic phase
3 following combined treatment with 25nM TDX and CH-11
4 (Fig. 2B). We also examined the effect of CH-11 on
5 apoptosis induced by the DNA-damaging agent OXA,
6 which also potently induces Fas expression in MCF-7
7 cells (Fig. 2C). Similar to its effect on 5-FU and
8 TDX-treated cells, CH-11 induced apoptosis of OXA-
9 treated cells, with ~50% of cells in the sub-G1/G0
10 apoptotic phase (Fig. 2D).

11
12 We subsequently analysed activation of the Fas
13 pathway in MCF-7 cells following co-treatment with
14 5-FU and CH-11. As already noted, treatment with 5-
15 FU alone resulted in dramatic up-regulation of Fas,
16 but had no effect on caspase 8 activation (Fig. 2E).
17 However, co-treatment of MCF-7 cells with 5-FU and
18 CH-11 resulted in a dramatic activation of caspase 8
19 as indicated by complete loss of procaspase 8 (Fig.
20 2E). Furthermore, cleavage of PARP (poly(ADP) ribose
21 polymerase), a hallmark of apoptosis, was only
22 observed in MCF-7 cells co-treated with 5-FU and CH-
23 11 (Fig. 2E). We next analysed the kinetics of
24 caspase 8 activation in 5-FU and CH-11 co-treated
25 cells. Caspase 8 was potently activated 12 hours
26 after addition of CH-11 to 5-FU pre-treated cells
27 (Fig. 2F). Importantly, this coincided with complete
28 processing of c-FLIP_L to its p43-form (Fig. 2F). By
29 24 hours after the addition of CH-11, neither
30 procaspase 8 nor c-FLIP_L (both its full-length and
31 truncated forms) was detected.

32

1 We also examined the ability of CH-11 to activate
2 apoptosis in the HCT116 colon cancer cell line. Fas
3 was potently up-regulated in HCT116 cells 48 hours
4 after treatment with 5-FU, TDX and OXA (Fig. 3A).
5 Treatment with each drug alone or CH-11 alone for 48
6 hours failed to significantly activate caspase 8 or
7 induce PARP cleavage (Fig. 3B). However, treatment
8 with each drug for 24 hours followed by CH-11 for a
9 further 24 hours resulted in activation of caspase 8
10 and PARP cleavage. Importantly, activation of
11 caspase 8 correlated with processing of c-FLIP_L in
12 drug and CH-11 co-treated cells (Fig. 3B).
13
14 Overexpression of c-FLIP_L inhibits chemotherapy-
15 induced Fas-mediated cell death. To further
16 investigate the role of c-FLIP_L in regulating Fas-
17 mediated apoptosis following drug treatment, we
18 developed a panel of MCF-7 cell lines overexpressing
19 c-FLIP_L. We developed cell lines with 5-10-fold
20 increased c-FLIP_L expression compared to cells
21 transfected with empty vector (Fig. 4A). The c-FLIP_L
22 -overexpressing cell lines FL44 and FL64 and cells
23 transfected with empty vector (EV68) were taken
24 forward for further characterisation. Cell viability
25 assays indicated that treatment of EV68 cells with
26 5-FU followed by CH-11 resulted in a highly
27 synergistic decrease in cell viability (RI=2.06,
28 $p<0.0005$) (Fig. 4B). However, no synergistic
29 decrease in cell viability was observed in 5-FU and
30 CH-11 co-treated FL44 or FL64 cells, with RI values
31 of 1.14 and 1.01 respectively (Fig. 4B).
32 Furthermore, 5-FU and CH-11 co-treatment resulted in

1 caspase 8 activation and PARP cleavage in EV68 cells
2 (Fig. 4C). In contrast, c-FLIP_L overexpression in
3 FL64 cells abrogated both activation of caspase 8
4 and PARP cleavage in response to 5-FU and CH-11 co-
5 treatment (Fig. 4C).

6
7 We next examined the effect of c-FLIP_L
8 overexpression on Fas-mediated apoptosis following
9 treatment with the antifolates TDX and MTA and the
10 DNA-damaging agent OXA. All three drugs
11 synergistically decreased cell viability in EV68
12 cells when combined with CH-11 (Figs. 5A and B).
13 However, this synergistic interaction was inhibited
14 by c-FLIP_L overexpression in both the FL44 and FL64
15 cell lines (Figs. 5A and B). Analysis of caspase 8
16 activation and PARP cleavage confirmed that Fas-
17 mediated apoptosis in response to all three agents
18 was attenuated by c-FLIP_L overexpression. Combined
19 treatment with each antifolate and CH-11 resulted in
20 caspase 8 activation in EV68 cells, but not FL64
21 cells (Fig. 5C). Similarly, PARP cleavage in
22 response to the antifolates and CH-11 was inhibited
23 in the FL64 cell line (Fig. 5C). Although some
24 caspase 8 activation and PARP cleavage were observed
25 in FL64 cells following co-treatment with 5µM OXA
26 and CH-11, this was much reduced compared to the
27 EV68 cell line (Fig. 5D). These results indicate
28 that c-FLIP_L is a key regulator of Fas-mediated
29 apoptosis in response to 5-FU, antifolates and
30 oxaliplatin.

31

1 siRNA-targeting of c-FLIP sensitises cancer cells to
2 chemotherapy. Having established that c-FLIP_L
3 overexpression protected MCF-7 and HCT116 cells from
4 chemotherapy-induced Fas-mediated cell death, we
5 next designed a FLIP-targeted (FT) siRNA to inhibit
6 both c-FLIP splice variants. Transfection with 10nM
7 FT siRNA potently down-regulated expression of both
8 c-FLIP splice variants in MCF-7 cells (Fig. 6A).
9 Cell viability analysis of MCF-7 cells transfected
10 with FT siRNA indicated that co-treatment with 5-FU
11 resulted in a supra-additive decrease in cell
12 viability (Fig. 6B, RI=1.56, $p<0.005$).
13 Interestingly, transfection of MCF-7 cells with FT
14 siRNA significantly decreased cell viability in the
15 absence of co-treatment with 5-FU, with an
16 approximate 50% decrease in cell viability in cells
17 transfected with 2.5nM FT siRNA (Fig. 6B). A
18 scrambled control (SC) siRNA that had no effect of
19 FLIP expression, also had no effect on cell
20 viability either alone or in combination with 5-FU
21 (data not shown). The decrease in cell viability in
22 response to FT siRNA alone appeared to be due to the
23 induction of apoptosis, as transfection of FT siRNA
24 in the absence of co-treatment with drug induced
25 significant levels of PARP cleavage (Fig. 6C, lane
26 2). Furthermore, combined treatment with FT siRNA
27 and 5-FU resulted in potent cleavage of PARP (Fig.
28 6C), indicating that the synergistic decrease in
29 cell viability observed in MCF-7 cells co-treated
30 with these agents was due to increased apoptosis.
31

1 FT siRNA also potently down-regulated FLIP_L and FLIP_S
2 expression in HCT116 cells (Fig. 7A). Analysis of
3 caspase 8 activation in siRNA-transfected HCT116
4 cells indicated that FT siRNA alone (1nM) caused
5 some activation of caspase 8, as indicated by the
6 decrease in the levels of p53/55 zymogen and
7 appearance of the p41/43 cleavage products (Fig. 7B,
8 lane 3). This was accompanied by some PARP cleavage.
9 At higher concentrations (>5nM), FT siRNA alone
10 caused more potent activation of caspase 8 and PARP
11 cleavage in HCT116 cells (Fig. 7C). Both 5-FU (5µM)
12 and TDX (100nM) caused some caspase 8 activation in
13 mock and SC transfected HCT116 cells as indicated by
14 the presence of p41/p43 caspase 8, although no PARP
15 cleavage was observed in these cells (Fig. 7B). The
16 most potent activation of caspase 8 was observed in
17 cells co-treated with 1nM FT siRNA and 5-FU or TDX,
18 with decreased expression of the p53/55 zymogen and
19 increased expression of both the p41/43 and p18
20 caspase 8 cleavage products (Fig. 7B, lanes 6 and
21 9). Furthermore, activation of caspase 8 in FT
22 siRNA/chemotherapy-treated HCT116 cells was
23 accompanied by potent PARP cleavage. Cell viability
24 assays indicated that co-treatment with 0.5nM FT
25 siRNA and 5µM 5-FU resulted in a synergistic
26 decrease in cell viability (Fig. 8A, RI=2.10,
27 $p<0.0005$). In contrast, SC siRNA had no significant
28 effect on cell viability either in the presence or
29 absence of 5-FU. Furthermore, co-treatment with FT
30 siRNA and both TDX and OXA resulted in synergistic
31 decreases in cell viability, with RI values of 1.68
32 and 2.26 respectively (Figs. 8B and C). These

1 results indicate that inhibition of c-FLIP
2 expression in HCT116 and MCF-7 cells dramatically
3 sensitised them to chemotherapy-induced apoptosis.

4
5
6 DISCUSSION

7
8 We found that the Fas death receptor was highly up-
9 regulated in response to 5-FU, the TS-targeted
10 antifolates TDX and MTA and the DNA-damaging agent
11 OXA in MCF-7 breast cancer and HCT116 colon cancer
12 cells, however, this did not result in significant
13 activation of apoptosis. Expression of FasL by
14 activated T cells and natural killer cells induces
15 apoptosis of Fas expressing target cells in vivo
16 (O'Connell et al., 1999). To mimic the effects of
17 these immune effector cells in our in vitro model,
18 we used the agonistic Fas monoclonal antibody CH-11.
19 We found that CH-11 potently activated apoptosis of
20 chemotherapy-treated cells, suggesting that the Fas
21 signalling pathway is an important mediator of
22 apoptosis in response to these agents in vivo. Many
23 tumour cells overexpress FasL, and it has been
24 postulated that tumour FasL induces apoptosis of
25 Fas-sensitive immune effector cells, thereby
26 inhibiting the antitumor immune response (O'Connell
27 et al., 1999). This hypothesis has been supported by
28 both in vitro and in vivo studies (Bennett et al.,
29 1998; O'Connell et al., 1997). The strategy of
30 overexpressing FasL requires that the tumour cells
31 develop resistance to Fas-mediated apoptosis to
32 prevent autocrine and paracrine induction of tumour

1 cell death. The lack of caspase 8 activation that we
2 observed in response to chemotherapy suggests that
3 Fas-mediated apoptosis may be inhibited in MCF-7 and
4 HCT116 and cancer cells, but that co-treatment with
5 CH-11 was sufficient to overcome this resistance and
6 activate Fas-mediated apoptosis.

7
8 Fas signalling may be inhibited by c-FLIP, which can
9 inhibit caspase 8 recruitment to and activation at
10 the Fas DISC (Krueger et al., 2001). Multiple c-FLIP
11 splice variants have been reported, however, only
12 two forms (c-FLIP_L and c-FLIP_S) have been detected at
13 the protein level (Scaffidi et al., 1999). Both
14 splice variants have death effector domains (DEDs),
15 with which they bind to FADD, blocking access of
16 procaspase 8 molecules to the DISC. c-FLIP_L is
17 processed at the DISC as it is a natural substrate
18 for caspase 8, which cleaves it to generate a
19 truncated form of approximately 43kDa (p43-FLIPL)
20 (Niikura et al., 2002). Cleaved p43- c-FLIP_L binds
21 more tightly to the DISC than full-length c-FLIP_L.
22 c-FLIP_S is not processed by caspase 8 at the DISC.
23 c-FLIP_L appears to be a more potent inhibitor of
24 Fas-mediated cell death than c-FLIP_S (Irmeler et al.,
25 1997; Tschopp et al., 1998). Initially both pro-
26 apoptotic and anti-apoptotic effects were proposed
27 for c-FLIP. However, enhanced cell death occurred
28 mainly in experiments using transient over-
29 expression and may have been due to excessive levels
30 of these DED-containing proteins, which may have
31 caused clustering of other DED-containing proteins
32 including procaspase 8, resulting in caspase

1 activation (Siegel et al., 1998). The data from cell
2 lines stably over-expressing c-FLIP and from mice
3 deficient in c-FLIP support an anti-apoptotic
4 function for c-FLIP (Yeh et al., 2000).

5

6 We found that c-FLIP_L was up-regulated and processed
7 to its p43-form in MCF-7 cells following treatment
8 with 5-FU and TDX. Furthermore, activation of
9 caspase 8 and apoptosis in cells co-treated with
10 chemotherapy and CH-11 coincided with processing of
11 c-FLIP_L. These results suggested that c-FLIP_L
12 regulated the onset of drug-induced Fas-mediated
13 apoptosis in these cell lines. This hypothesis was
14 further supported by data from overexpression and
15 siRNA studies. c-FLIP overexpression abrogated the
16 synergistic interaction between CH-11 and 5-FU, TDX,
17 MTA and OXA by inhibiting caspase 8 activation.
18 Furthermore, siRNA-targeting of both c-FLIP splice
19 variants sensitised cells to these chemotherapeutic
20 agents as determined by cell viability and PARP
21 cleavage assays. Collectively, these results
22 indicate that c-FLIP inhibits apoptosis in response
23 to these drugs.

24

25 Interestingly, we also found that siRNA-mediated
26 down-regulation of c-FLIP_L and c-FLIP_S induced
27 caspase 8 activation and PARP cleavage in the
28 absence of co-treatment with chemotherapy (although
29 co-treatment with drug enhanced the effect). The
30 mechanism of FLIP-targeted siRNA-mediated activation
31 of apoptosis is currently being investigated. In
32 addition to blocking caspase 8 activation, DISC-

1 bound c-FLIP has been reported to promote activation
2 of the ERK, PI3-kinase/Akt and NFkB signalling
3 pathways (Kataoka et al., 2000; Panka et al., 2001).
4 The NFkB, PI3K/Akt and ERK signal transduction
5 pathways are associated with cell survival and/or
6 proliferation, therefore, c-FLIP is capable of both
7 blocking caspase 8 activation and also recruiting
8 adaptor proteins that can activate intrinsic
9 survival and proliferation pathways (Shu et al.,
10 1997). Furthermore, c-FLIP also inhibits procaspase
11 8 activation at the DISCs formed by the TRAIL
12 receptors DR4 and DR5 (Krueger et al., 2001). rTRAIL
13 induces apoptosis in a range of human cancer cell
14 lines including colorectal and breast, indicating
15 that the TRAIL receptors are widely expressed in
16 tumour cells (Ashkenazi, 2002). It is possible that
17 expression of DR4 and DR5 is tolerated in tumours
18 because c-FLIP converts the apoptotic signal to one
19 which promotes survival and proliferation. Thus,
20 siRNA-mediated down-regulation of c-FLIP may induce
21 apoptosis by inhibiting FLIP-mediated activation of
22 NFkB, PI3K/Akt and ERK and promoting activation of
23 caspase 8 at TRAIL DISCs.

24

25 In conclusion, we have found that c-FLIP is a key
26 regulator of Fas-mediated apoptosis in response to
27 5-FU, TS-targeted antifolates and OXA. Our results
28 suggest that c-FLIP may be a clinically useful
29 predictive marker of response to these agents and
30 that c-FLIP is a therapeutically attractive target.

31

32 All documents referred to in this specification are

1 herein incorporated by reference. Various
2 modifications and variations to the described
3 embodiments of the inventions will be apparent to
4 those skilled in the art without departing from the
5 scope and spirit of the invention. Although the
6 invention has been described in connection with
7 specific preferred embodiments, it should be
8 understood that the invention as claimed should not
9 be unduly limited to such specific embodiments.
10 Indeed, various modifications of the described modes
11 of carrying out the invention which are obvious to
12 those skilled in the art are intended to be covered
13 by the present invention.

14

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10

11

12

1 Claims

2

3 1. A method to predict response of tumour cells
4 to in vivo treatment with a chemotherapeutic
5 regime, said method comprising the steps:
6 (a) providing an in vitro sample containing
7 tumour cells from a subject;
8 (b) determining the basal expression of one or
9 more of the genes encoding c-FLIP protein,
10 wherein enhanced expression of said gene
11 correlates with enhanced resistance to the
12 chemotherapeutic regime.

13

14 2. A method for evaluating in vitro the response
15 of tumour cells from a subject to the presence
16 of a chemotherapeutic regime to predict
17 response of the tumour cells in vivo to
18 treatment with the chemotherapeutic regime,
19 which method comprises:
20 (a) providing an in vitro sample containing
21 tumour cells from a subject;
22 (b) exposing a portion of said sample of
23 tumour cells to said chemotherapeutic regime;
24 (c) measuring expression of c-FLIP in said
25 tumour cells; wherein enhanced expression of
26 c-FLIP in response to said chemotherapeutic
27 regime is indicative of enhanced resistance to
28 said chemotherapeutic regime.

29

30 3. The method according to claim 1 or claim 2,
31 wherein the chemotherapeutic regime comprises
32 treatment using a death receptor ligand

- 1 combined with a chemotherapeutic agent.
2
- 3 4. The method according to claim 3 wherein the
4 death receptor ligand is CH-11 and the
5 chemotherapeutic agent is 5-FU or an
6 antifolate drug.
7
- 8 5. A method of sensitising cancer cells to
9 chemotherapy, said method comprising the step
10 of administration to said cells a c-FLIP
11 inhibitor.
12
- 13 6. An assay method for identifying a
14 chemotherapeutic agent for use in the
15 treatment of cancer, said method comprising
16 the steps:
17 (a) providing a sample of tumour cells;
18 (b) exposing a portion of said sample to a
19 candidate chemotherapeutic agent;
20 (c) determining expression of c-FLIP in said
21 sample wherein a reduction in expression of c-
22 FLIP compared to expression in a control
23 sample is indicative of chemotherapeutic
24 activity.
25
- 26 7. A method of killing cancer cells comprising
27 administration of a therapeutically effective
28 amount of a c-FLIP inhibitor.
29
- 30 8. A method of treating cancer comprising
31 administration of a therapeutically effective

- 1 amount of a c-FLIP inhibitor.
2
- 3 9. The method according to any one of claims 5 to
4 8, wherein the c-FLIP inhibitor is
5 administered as part of a treatment regime
6 comprising
7 (a) a c-FLIP inhibitor and
8 (b) (i) a specific binding member which binds
9 to a cell death receptor, or a nucleic acid
10 encoding said binding member; and
11 (ii) a chemotherapeutic agent.
12
- 13 10. The method according to claim 9, wherein the
14 binding member is the FAS antibody CH11.
15
- 16 11. The method according to claim 9 or claim 10
17 wherein the chemotherapeutic agent is 5-FU or
18 an antifolate.
19
- 20 12. The use of a c-FLIP inhibitor in the
21 preparation of a medicament for treating
22 cancer.
23
- 24 13. The use of
25 (a) a c-FLIP inhibitor and
26 (b) (i) a specific binding member which binds
27 to a cell death receptor, or a nucleic acid
28 encoding said binding member; and/or
29 (ii) a chemotherapeutic agent in the
30 preparation of a medicament for treating
31 cancer.
32

- 1 14. The use according to claim 13, wherein the
2 binding member is the FAS antibody CH11.
3
- 4 15. The use according to claim 13 or claim 14
5 wherein the chemotherapeutic agent is 5-FU or
6 an antifolate.
7
- 8 16. A pharmaceutical composition for the treatment
9 of cancer, wherein the composition comprises a
10 c-FLIP inhibitor and a pharmaceutically
11 acceptable excipient, diluent or carrier.
12
- 13 17. The pharmaceutical composition according to
14 claim 11 wherein wherein the composition
15 comprises a) a c-FLIP inhibitor and
16 (b) (i) a specific binding member which binds
17 to a cell death receptor, or a nucleic acid
18 encoding said binding member; and/or
19 (ii) a chemotherapeutic agent and
20 (c) a pharmaceutically acceptable excipient,
21 diluent or carrier.
22
- 23 18. The pharmaceutical composition according to
24 claim 17, wherein the binding member is the
25 FAS antibody CH11.
26
- 27 19. The pharmaceutical composition according to
28 claim 17 or claim 18 wherein the
29 chemotherapeutic agent is 5-FU or an
30 antifolate.
31

- 1 20. A kit for the treatment of cancer, said kit
2 comprising a) a c-FLIP inhibitor and
3 (b) (i) a specific binding member which binds
4 to a cell death receptor, or a nucleic acid
5 encoding said binding member; and/or
6 (ii) a chemotherapeutic agent and
7 (c) instructions for the administration of (a)
8 and (b) separately, sequentially or
9 simultaneously.
- 10
- 11 21. The kit according to claim 20, wherein the
12 binding member is the FAS antibody CH11.
- 13
- 14 22. The kit according to claim 20 or claim 21
15 wherein the chemotherapeutic agent is 5-FU or
16 an antifolate.
- 17
- 18 23. The method according to any one of claims 5 to
19 11, the use according to any one of claims 12
20 to 15, the pharmaceutical composition
21 according to any one of claims 16 to 19 or the
22 kit according to any one of claims 20 to 22,
23 wherein said c-FLIP inhibitor is an RNAi
24 agent, which modulates expression of a c-FLIP
25 gene.
- 26
- 27 24. The method according to any one of claims 5 to
28 11, the use according to any one of claims 12
29 to 15, the pharmaceutical composition
30 according to any one of claims 16 to 19 or the
31 kit according to any one of claims 20 to 22,
32 wherein the c-FLIP inhibitor an RNAi agent

74

1 having nucleotide sequence
2 AAG CAG TCT GTT CAA GGA GCA (SEQ ID NO: 1).
3
4

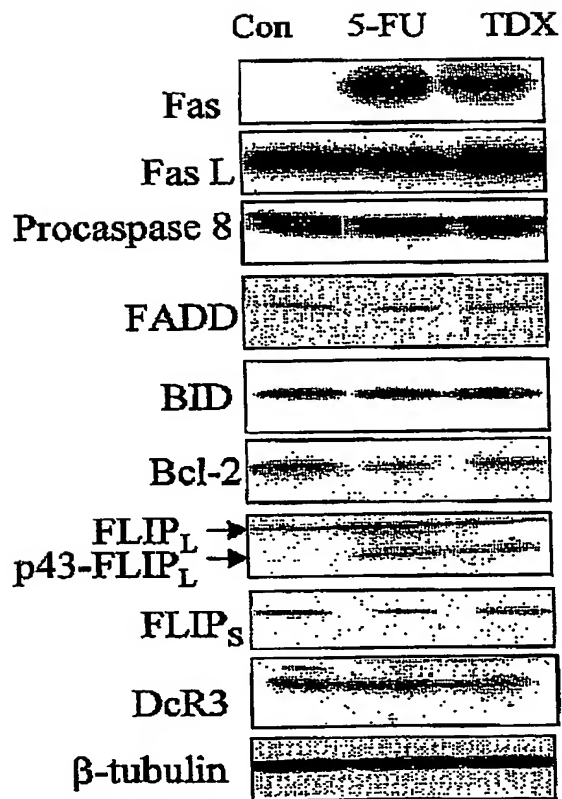


Figure 1A

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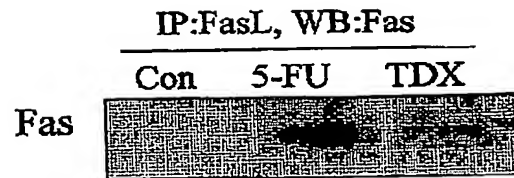


Figure 1B

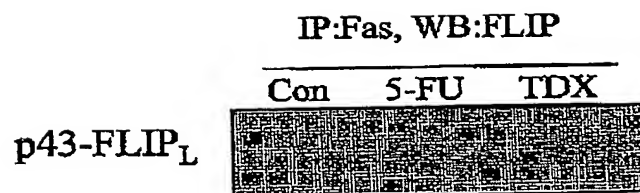
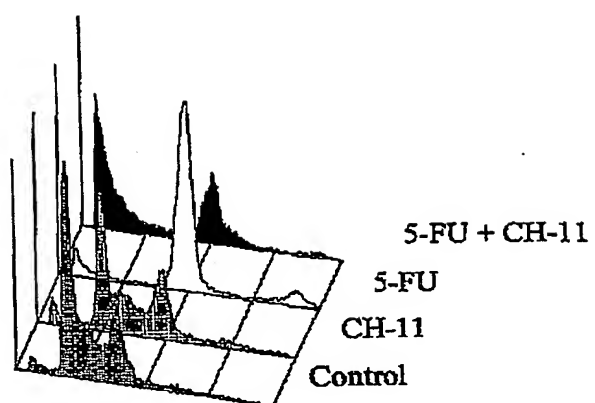


Figure 1C

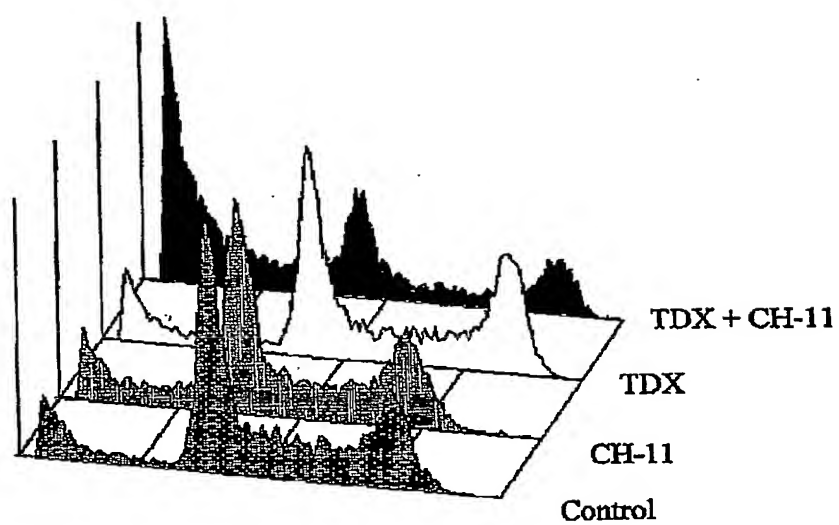
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Figure 2 A



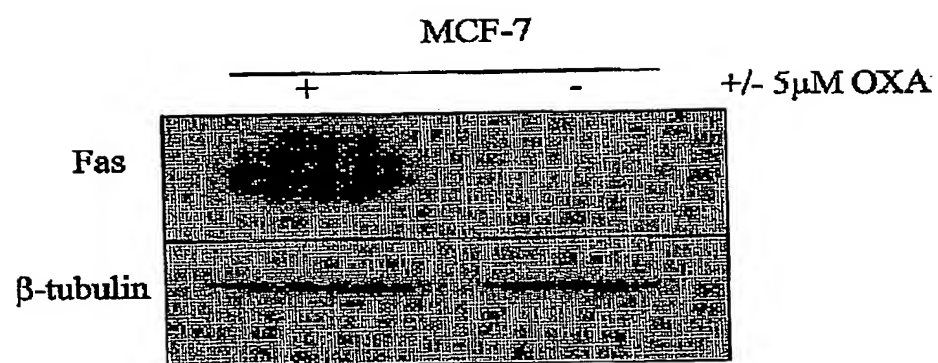
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Figure 2B



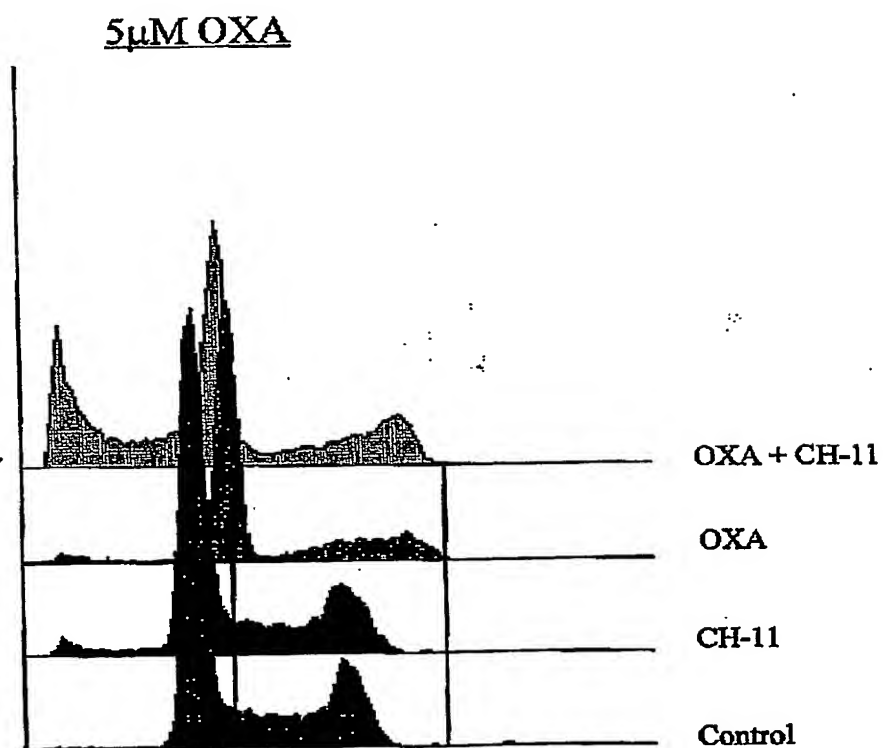
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Figure 2C



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Figure 2D



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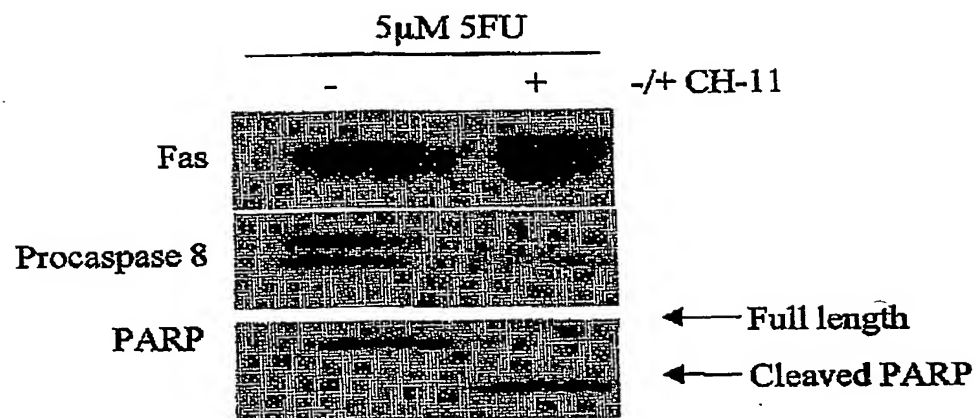


Figure 2E

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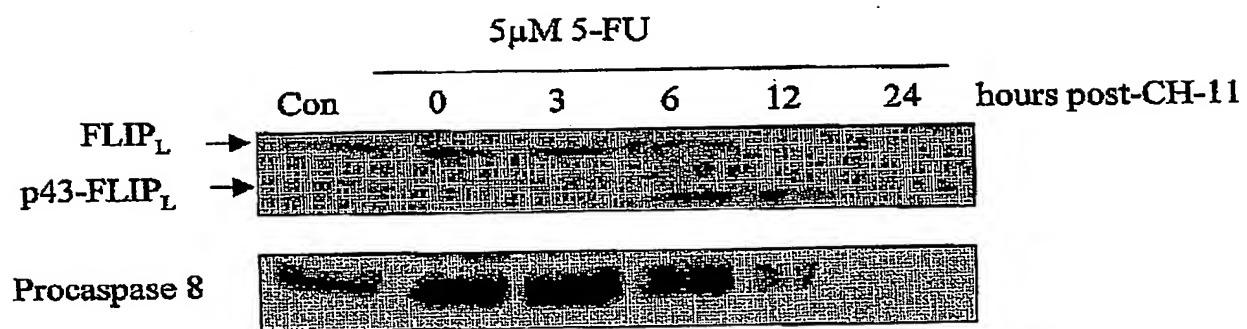


Figure 2F

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Figure 3A

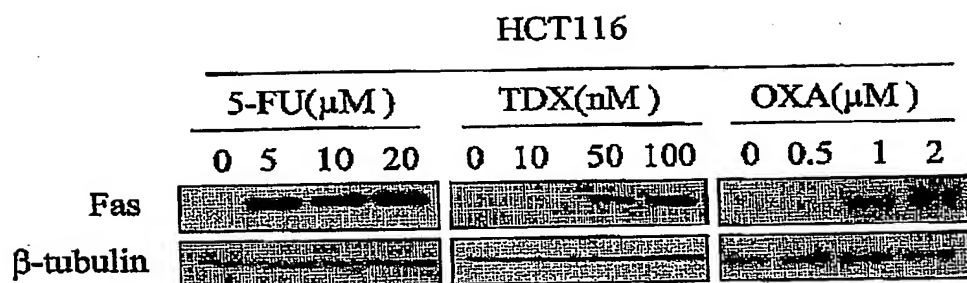
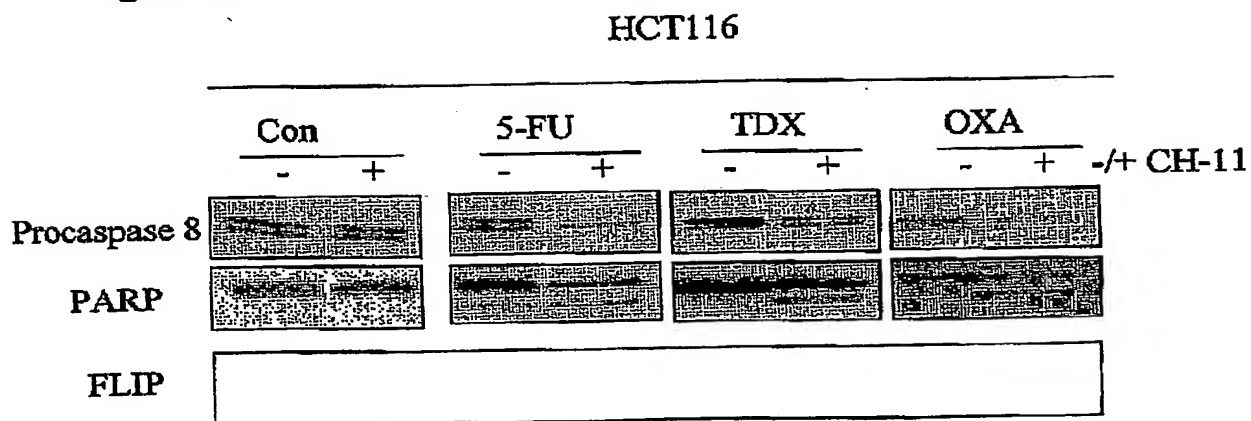


Figure 3B



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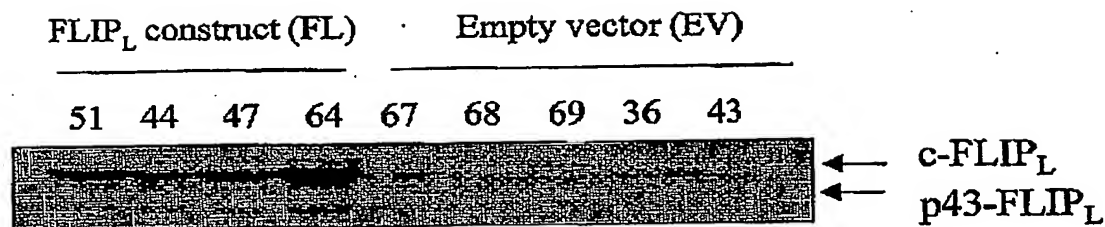


Figure 4A

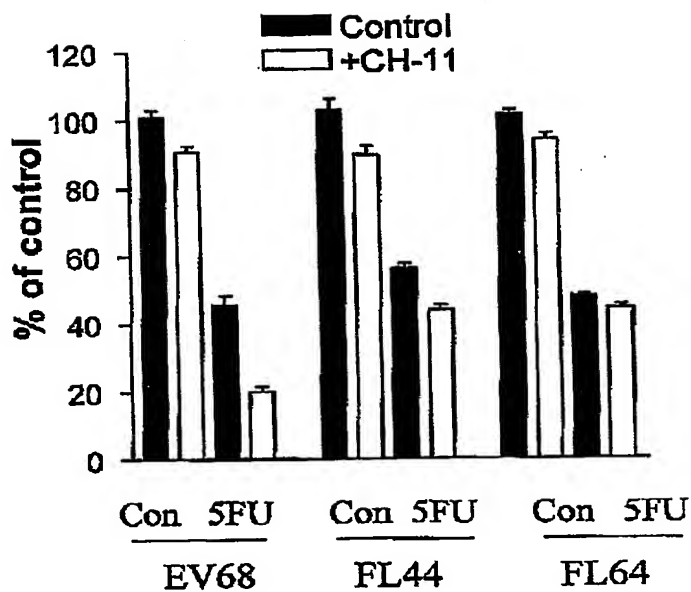


Figure 4B

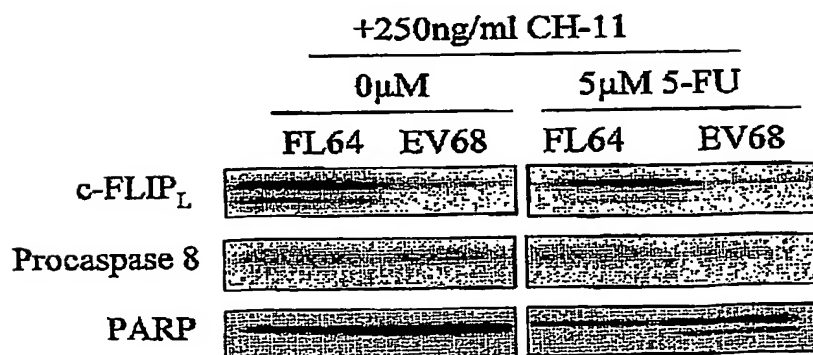


Figure 4C

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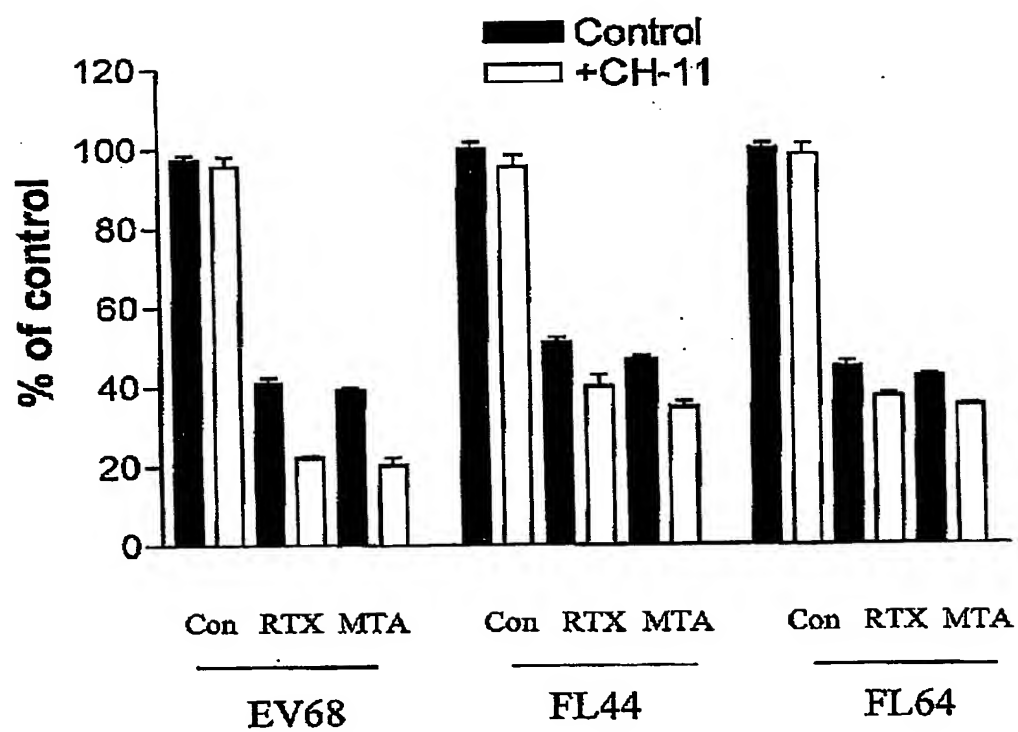


Figure 5A

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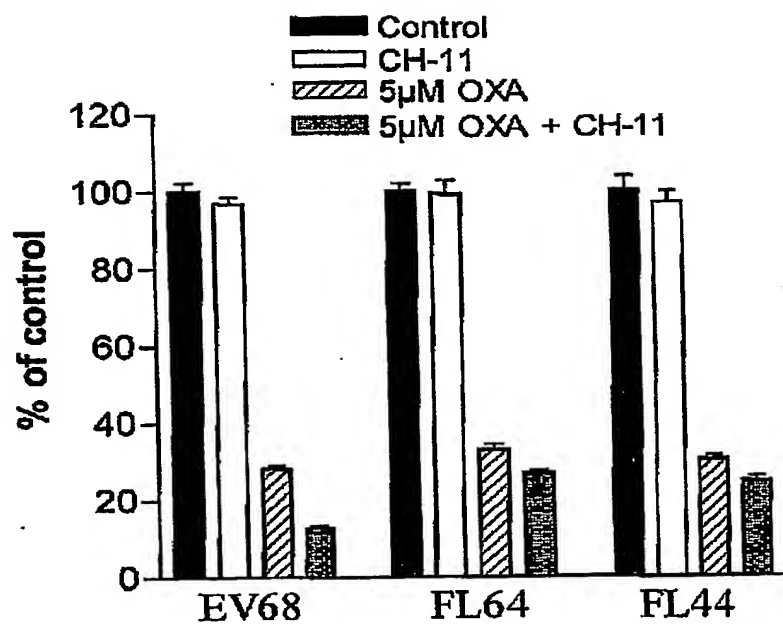


Figure 5B

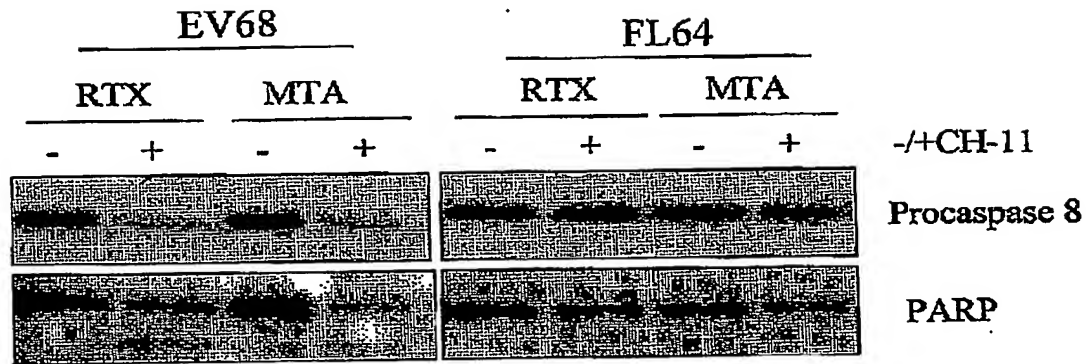


Figure 5C

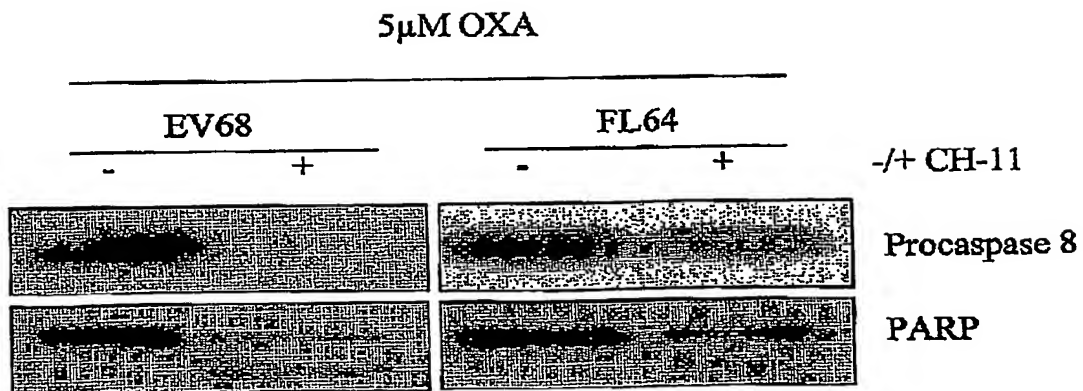


Figure 5D

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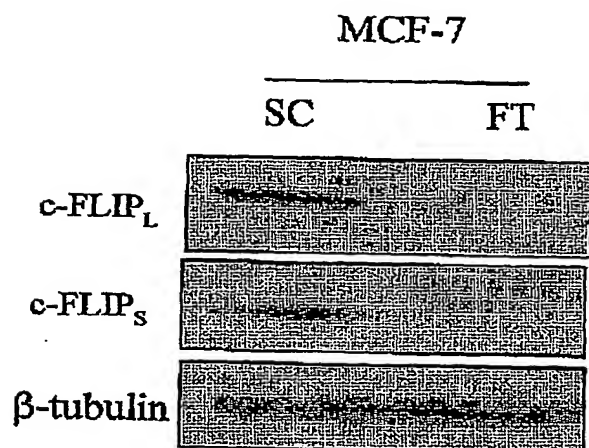


Figure 6A

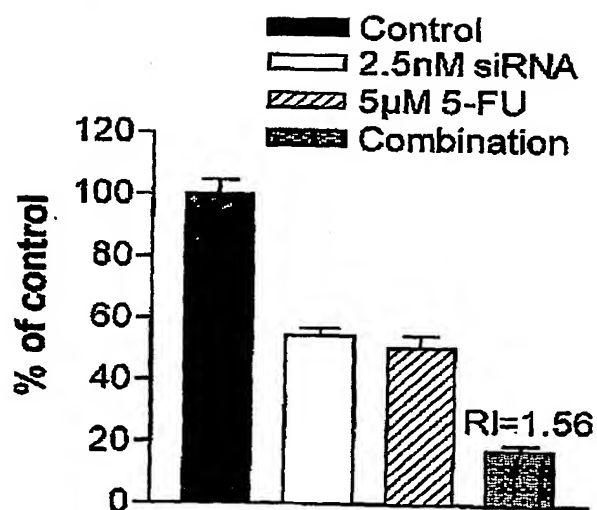
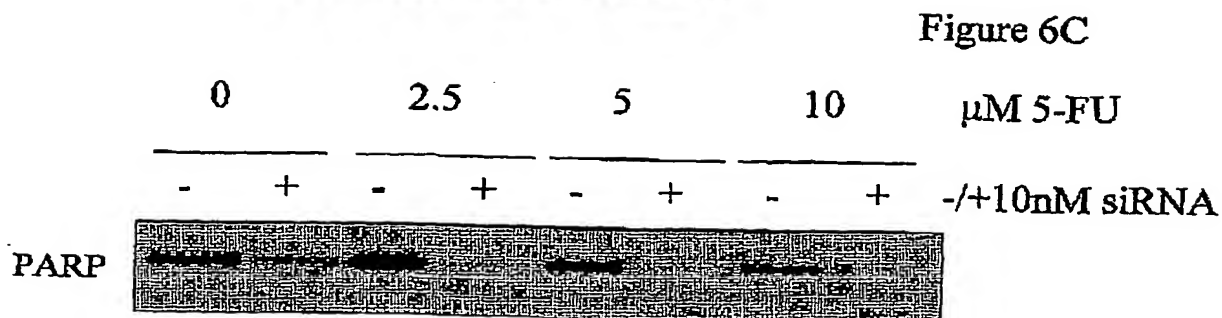


Figure 6B



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Figure 7A

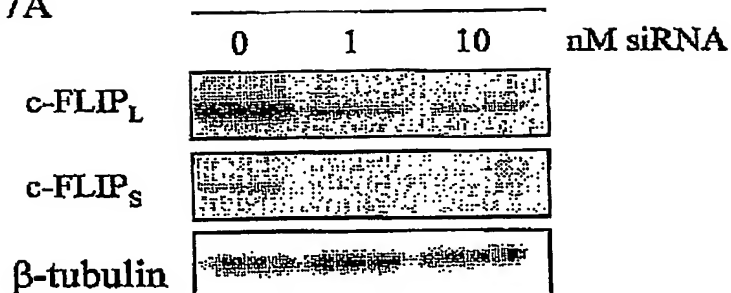
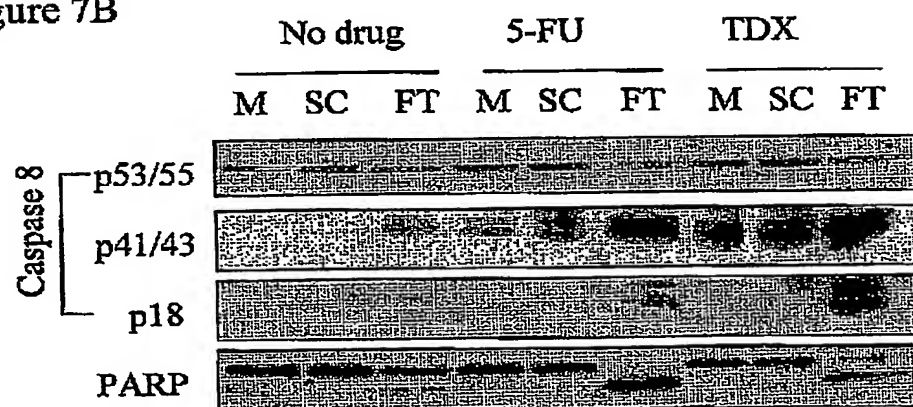
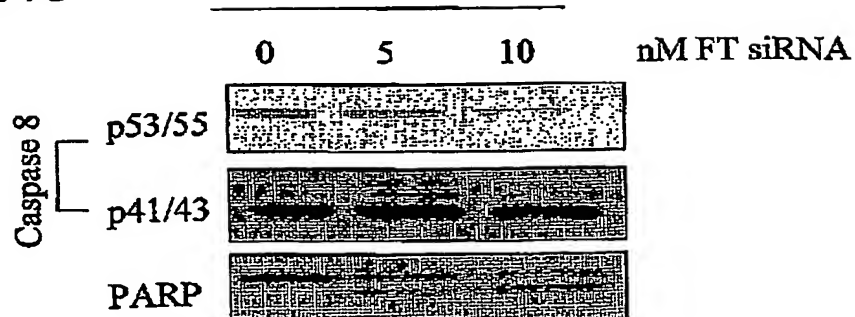


Figure 7B



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Figure 7C



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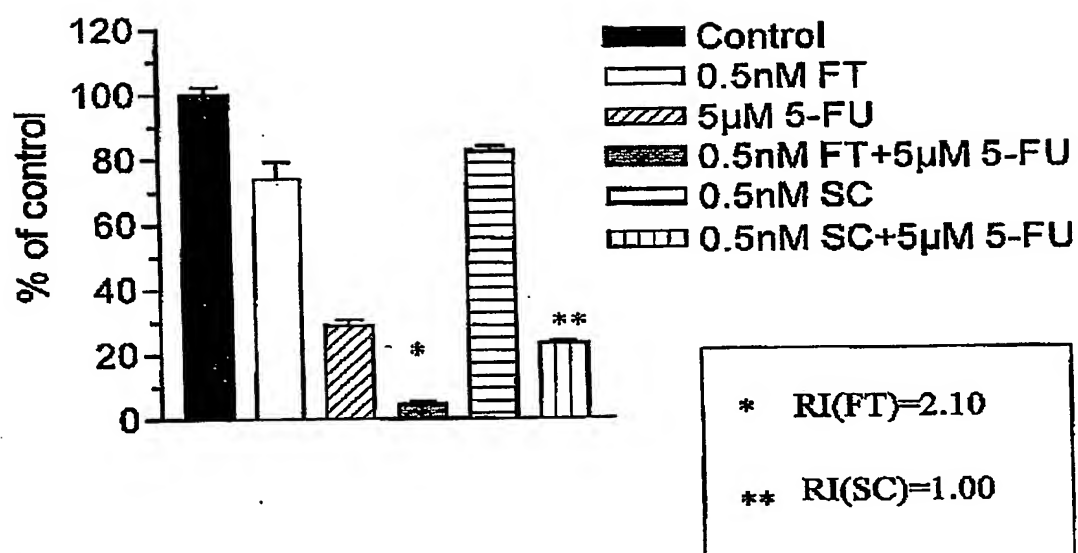
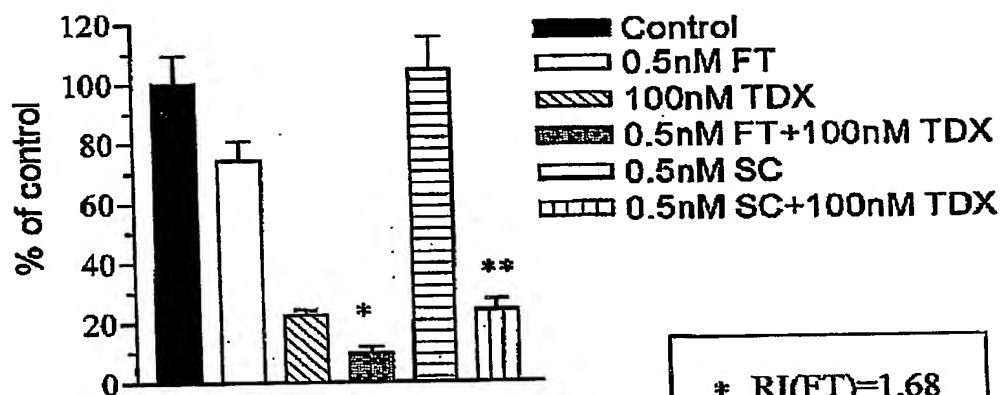


Figure 8A

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* RI(FT)=1.68

** RI(SC)=0.91

Figure 8B

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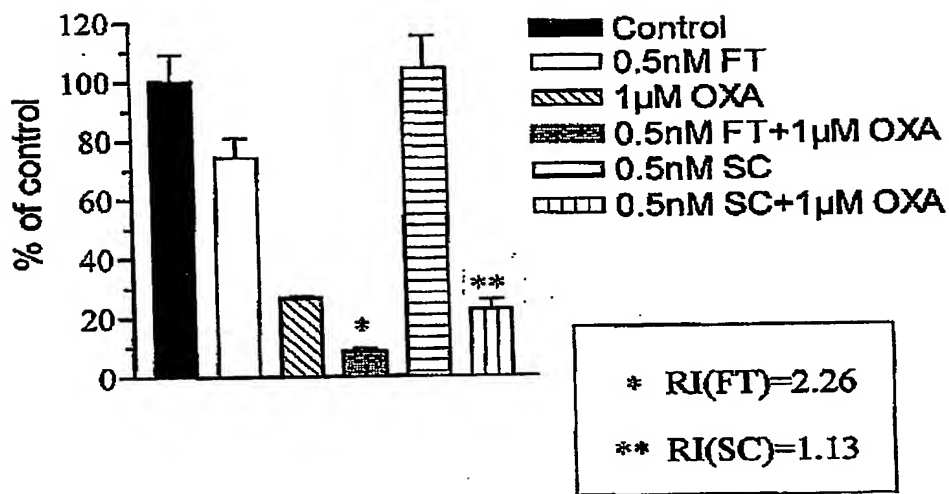
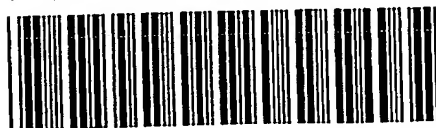


Figure 8C

PCT/GB2004/005006



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